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Inoculation of endophytic bacteria on host and non-host plants—Effects on plant growth and Ni uptake

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ABSTRACT

Among a collection of Ni resistant endophytes isolated from the tissues of *Alyssum serpyllifolium*, four plant growth promoting endophytic bacteria (PGPE) were selected based on their ability to promote seedling growth in roll towel assay. Further, the PGPE screened showed the potential to produce plant growth promoting (PGP) substances and plant polymer hydrolyzing enzymes. These isolates were further screened for their PGP activity on *A. serpyllifolium* and *Brassica juncea* under Ni stress using a phytagar assay. None of the four isolates produced any disease symptoms in either plant. Further, strain A3R3 induced a maximum increase in biomass and Ni content of plants. Based on the PGP potential in phytagar assay, strain A3R3 was chosen for studying its PGP effect on *A. serpyllifolium* and *B. juncea* in Ni contaminated soil. Inoculation with A3R3 significantly increased the biomass (*B. juncea*) and Ni content (*A. serpyllifolium*) of plants grown in Ni contaminated soil. The strain also showed high level of colonization in tissue interior of both plants. By 16S rRNA gene sequencing analysis, A3R3 was identified as *Pseudomonas* sp. Successful colonization and subsequent PGP potentiality of *Pseudomonas* sp. A3R3 indicate that the inoculation with PGPE might have significant potential to improve heavy metal phytoremediation.

1. Introduction

Industrial operations such as mining, smelting, metal forging, manufacturing of alkaline storage batteries, combustion of fossil fuel, and sewage sludge, cause accumulation of metals or metalloids in natural resources such as soil, water and air. Since the heavy metals seriously affect terrestrial and aquatic ecosystems and induce potential health risks [1], various physicochemical and biological methods have been developed to remove the metals from the environment. Phytoremediation refers to the use of plants that can uptake high levels of heavy metals from soil and accumulate them in a harvestable part [2]. Although the efficiency of heavy metal phytoremediation is dependent on an adequate yield of plants and their capacity for metal ion accumulation, the plant associated beneficial microbes also play significant roles as they can provide nutrients and reduce the deleterious effects of metals to the plants [3,4]. Considering such beneficial features, it may be envisaged that inoculation of metal resistant plant growth promoting bacteria would increase plant growth and phytoremediation potential in metal contaminated soils [5–7].

In recent years, the use of metal resistant endophytic bacteria in phytoremediation of heavy metal contaminated soils has attracted more attention [8–11]. Although the heavy metals such as Ni, Pb are toxic to plants and their associated microbes at high concentrations, the metal resistant plant growth promoting endophytic bacteria (PGPE) have been reported to occur widely in tissue interiors of various hyperaccumulator plants [12–14]. This indicates that endophytic bacteria have evolved to be resistant to high levels of heavy metals and that they might confer to the plant higher tolerance to heavy metal stress. Moreover, the endophytic bacteria are proved to be able to enhance the plant growth by various mechanisms including production of siderophores, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, indole-3-acetic acid (IAA) or solubilization of phosphate (P) [15]. In addition, certain endophytes have also been shown to alter heavy metal toxicity/availability to the plant by producing siderophores, biosurfactants and organic acids [10,16]. Despite these beneficial actions on plant, however, the endophytic bacteria must be compatible with various hyperaccumulators and able to colonize the tissues of the host plants without producing any disease symptoms. Because of the ability to produce the plant growth beneficial substance in metal stressed environment, the colonization potential of PGPE in the rhizosphere and/or tissue interior of plants has been considered as a major

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factor that determines the inoculum efficiency for microbial assisted heavy-metal phytoremediation [4,10].

Although the endophytic bacteria are reported to be present in various plants growing on heavy metal contaminated soils, only a few attempts have been made to study their role on the growth and Ni accumulation in plants. Moreover, the role of PGPE on the growth and phytoremediation potential of a non-host plant in metal contaminated soils has not been adequately defined. The objectives of our study were (1) to isolate and characterize Ni resistant PGPE from the tissues of the Ni hyperaccumulator plant, *Alyssum serpyllifolium* ssp. *lusitanicum*, (2) to test whether the PGPE with known plant growth promoting (PGP) traits promote the plant growth and Ni accumulation in their host (*A. serpyllifolium*) and non-host plants (*Brassica juncea* L. Czern.), and (3) to select a Ni resistant PGPE strain which might be useful to increase the plant Ni uptake and biomass production for improving the efficiency of phytoremediation of Ni-contaminated soils.

2. Materials and methods

2.1. Isolation of Ni resistant PGPE

Endophytic bacteria were isolated from leaves, stems and roots of Ni accumulators, *A. serpyllifolium* grown in serpentine soils in Bragança, north-east of Portugal, previously described by Freitas et al. [17]. Briefly, plant samples were washed with tap water followed by three rinses with deionized water and then separated into roots, stems and leaves. Healthy plant tissues were sterilized by sequential immersion in 70% (v/v) ethanol for 1 min, and 3% sodium hypochlorite for 3 min and washed three times with sterile water to remove surface sterilization agents. In order to confirm the surface disinfection process was successful, sterility was checked by plating on Luria–Bartani (LB) agar. No contamination was found. After surface sterilization, the leaf, stem or root tissue was cut and titrated in distilled water; appropriate dilutions were plated onto sucrose-minimal salts low-phosphate (SLP) agar medium (sucrose 1%; $(NH_4)_2SO_4$ 0.1%; K_2HPO_4 0.05%; $MgSO_4$ 0.05%; NaCl 0.01%; yeast extract 0.05%; $CaCO_3$ 0.05%; pH 7.2) amended with 50 mg of Ni L^{-1} ($NiCl_2$). To isolate Ni resistant strains, the bacterial strains picked from the Ni resistant colonies were purified on the LB agar medium containing 50 mg L^{-1} of Ni and gradually taken to higher concentration of Ni (100–1000 mg L^{-1}) according to the procedure of Ma et al. [18].

In order to isolate the Ni resistant PGPE, the Ni resistant strains were assessed for the PGP activity by roll towel method [19]. Seeds of *A. serpyllifolium* obtained from the Botanical Garden of the University of Coimbra, Coimbra, Portugal, were surface sterilized in 2% $Ca(OCl)_2$ (2 h) and rinsed several times with sterile distilled water. The seeds were inoculated by soaking in a bacterial suspension containing 10^8 cell mL^{-1} for 2 h then placed in wet blotters and incubated in a growth chamber for 30 days. The vigour index was calculated as described by Abdul-Baki and Anderson [20].

2.2. Characterization of PGP traits of endophytic bacteria

To determine ACC deaminase activity, the PGPE were grown in test tubes containing 10 mL of Dworkin–Foster (DF) salts minimal medium [21]. The medium was supplemented with 3 mM ACC. After cultivation for 72 h at 27 °C, the cells were harvested by centrifugation at 9000 rpm for 10 min at room temperature. The ACC deaminase activity in cells was determined by monitoring the amount of α-ketobutyrate generated by the enzymatic hydrolysis of ACC as described by Belimov et al. [22]. The protein concentration of cell suspensions was determined by the method of Bradford [23].

IAA production by PGPE was determined as described previously [24]. Cultures of the isolates were raised in LB broth amended with 500 μg of tryptophan mL^{-1} at 27 °C for 96 h at 200 rpm. Bacterial cells were removed by centrifugation at 7000 rpm and the supernatant was analyzed for IAA.

The phosphate solubilizing activity of the isolates was analyzed in modified Pikovskayas medium [25] amended with tricalcium phosphate. The isolates were grown at 27 °C for 192 h at 200 rpm. The solubilized phosphate in the culture supernatant was quantified as described by Fiske and Subbarow [26].

Siderophores production by PGPE was determined using in chrome azurol S (CAS) agar medium [27]. The presence of catechol and hydroxamate type siderophores in culture supernatants obtained from bacteria grown under iron-limiting conditions in casamino acids (CAA) medium was quantitatified according to the method of Ma et al. [18].

2.3. Extracellular enzyme activities

Cellulase and pectinase activities were assayed on the indicator plates. For the cellulase assay, nitrogen-freebase (NFB) [28] plates supplemented with 0.2% carboxymethyl cellulose and 0.5% tryptone were spotted with bacterial cells. After incubating for 48 h at 30 °C the plates were flooded with congo red (1 mg mL^{-1}) solution for 30 min. Excess stain was discarded and the agar was destained with 1 M of NaCl solution [29]. Plates were kept overnight at 4 °C and examined for clearing zone around the point of inoculations. For the pectinase assay, the bacterial isolates were spotted on nutrient agar supplemented with 0.5% pectin. After incubating the plates for 5 days at 30 °C, the surface of the medium was overlayed with 2% hexadecyl trimethyl ammonium bromide (CTAB) solution for 30 min. CTAB solution was then discarded and the plate surface was washed with 1 M NaCl to visualize the zone around the bacterial growth [30].

2.4. Phytagar assay

This experiment was carried out to screen the Ni resistant PGPE for their ability to promote the growth and Ni accumulation of *A. serpyllifolium* and *B. juncea* growing in Ni treated agar media (5 mg Ni L^{-1}). The growth media were prepared using 0.5% phytagar and one-quarter strength Hoagland's nutrient solution with or without Ni. The surface sterilized seeds of *A. serpyllifolium* and *B. juncea* were inoculated with PGPE as detailed in earlier section and placed in 150 mL test tubes containing 25 mL of phytagar. The tubes were closed, placed in a growth chamber, and harvested after 60 days. At harvest, the plants were removed from the boxes and rinsed thoroughly in distilled water to remove adhering agar. Growth parameters such as fresh weight and dry weight of the plants were measured. The total Ni accumulation in plants was also determined [17].

2.5. Pot experiment

For pot experiments, the soil was collected from the Botanical Garden of the University of Coimbra, Coimbra, Portugal. The physicochemical properties of soil were: pH (1:1, w/v water) 7.3; organic matter 1.6%; nickel 18 mg kg^{-1}; zinc 86 mg kg^{-1}; chromium 31 mg kg^{-1}. The soil was sieved (2 mm) and sterilized by steaming (100 °C for 1 h on three consecutive days). After sterilization the soil was amended with aqueous solution of $NiCl_2$ to achieve the final concentrations of 150, 300 or 450 mg Ni kg^{-1} and left in a greenhouse for a 2-week period (for metal stabilization). Before inoculation, the mutant of A3R3 marked with antibiotic resistance was obtained after plating of the parental strain onto LB agar amended with ampicillin (75 mg L^{-1}). The surface sterilized seeds

of *A. serpyllifolium* ssp. lusitanicum and *B. juncea* L. Czern. were inoculated with A3R3 as detailed in earlier section. Seeds soaked in sterile water were used as control. The inoculated and non-inoculated seeds were planted in plastic pot containing 300 g of soil. The plants were grown in a greenhouse at 25 °C and a 16/8 day/night regime. After 60 days, the plants were carefully removed from the pots and the root surface was cleaned several times with distilled water. Plant fresh weight and dry weight were measured. The accumulation of Ni in root and shoot system was quantified as described above. The population dynamics of introduced bacteria was also studied using the intrinsic antibiotic marker. The plant interior colonization was quantified according to the procedure of the above endophytic bacterial isolation. The resulting suspensions were evaluated for colony forming units (CFU) according to the dilution-plate method on LB agar with addition of 75 mg L^{-1} ampicillin. The plates were incubated for 4 days at 28 °C.

2.6. Genetic characterization of Ni-resistant PGPE

For genotypic characterization, the PGPE strain was grown in LB medium in presence of 1 mM Ni for 20 h and total DNA was isolated using standard procedure [31]. The 16S rDNA was amplified using the primers pA (5′-AGAGTTTGATCCTGGCTCAG; *Escherichia coli* bases 8–27) and pC5B (5′-TACCTTGTTACGACTT; *E. coli* bases 1507–1492) [32] under the reaction conditions described by Branco et al. [33]. Partial nucleotide sequence of the amplified 16S rDNA was determined using automated DNA sequencer. The sequences obtained were matched against nucleotide sequences present in GenBank using the BLASTn program [34].

2.7. Statistical analysis

Analysis of variance (ANOVA) followed by post hoc Fisher Least Significant Difference (LSD) test ($p < 0.05$) were used to compare treatment means. All the statistical analyses were carried out using SPSS 10.0.

3. Results and discussion

3.1. Isolation of Ni resistant PGPE

The plants growing in metal contaminated soils accumulate higher amounts of heavy metals and can therefore provide a metal-stressed environment for endophytes where they can develop mechanisms to resist the toxic effects of metals [13]. Moreover, these metal resistant endophytes promote host plant growth by improving mineral nutrition or conferring tolerance to various biotic and abiotic stresses [15]. During the initial screening (50 mg L^{-1}), 95 Ni resistant bacterial strains were isolated from root, stem and leaf tissues of *A. serpyllifolium*. After secondary screening, 27 bacterial strains showing a high degree of Ni-resistance were selected (data not shown). In order to isolate the PGPE, the metal resistant strains were assessed for PGP activity on *A. serpyllifolium* by roll towel method. Among the 27 strains tested, four isolates, namely A3R3, A3S4, A2R6 and A3S6 induced an increase in root length, shoot length and vigour index of *A. serpyllifolium* (Table 1). However, A3R3 induced a maximum increase in root length, shoot length and vigour index by 32%, 45% and 55%, respectively, compared with non-inoculated control.

Table 1
Influence of Ni resistant endophytes on root length, shoot length and vigour index of *A. serpyllifolium*.

Treatment	Shoot length (cm)	Root length (cm)	Vigour index
Control	2.0 ± 0.2 b	1.9 ± 0.3 b	312.0 ± 21.2 c
A3R3	2.9 ± 0.2 a	2.5 ± 0.2 a	483.0 ± 27.5 a
A3S4	2.5 ± 0.2 a	2.3 ± 0.2 ab	408.0 ± 22.5 b
A2R6	2.6 ± 0.2 a	2.2 ± 0.3 ab	402.3 ± 41.9 b
A3S6	2.6 ± 0.3 a	2.3 ± 0.2 ab	422.2 ± 19.6 b

Average ± standard deviation from three samples. Data of columns indexed by the same letter are not significantly different between Ni resistant endophyte treatments according to Fisher's protected LSD test ($p < 0.05$).
Vigour index = germination (%) × seedling length (shoot length + root length).

3.2. Characterization of Ni resistant PGPE

Since the PGPE could exert their beneficial effects on host plant by several mechanisms including nitrogen fixation, phosphate solubilization, IAA and siderophore production [35], the PGP traits of Ni resistant PGPE were further investigated in detail. Assessment of the parameters of PGP revealed the intrinsic ability of the Ni resistant PGPE for the utilization of ACC as the sole nitrogen source, production of IAA, siderophore and solubilization of phosphate (Table 2). The role of ACC deaminase in decreasing stress ethylene levels by the enzymatic hydrolysis of ACC into α-ketobutyric acid and ammonia has been presented as one of the major mechanisms of PGPE in promoting root and plant growth [36]. Among the four strains tested, strain A3R3 recorded the highest ACC deaminase activity followed by A3S4. Another important PGP mechanism is the solubilization of P, by which microbes enhance P availability to the host plant [37]. The strains A3R3 and A3S6 showed the phosphate solubilizing ability by utilizing the insoluble tricalcium phosphate in modified Pikovskayas medium. Further screening of the production of IAA by Ni resistant PGPE indicated that all the four strains utilized L-tryptophan as a precursor for growth and IAA production. However, strain A3R3 produced the highest amount, 69.4 mg L^{-1} of IAA, whereas A2R6 produced only 43.8 mg L^{-1} of IAA. The IAA released by bacteria enhances plant growth directly by stimulating elongation of the cell or affecting cell division [38]. Besides, A3R3, A3S4 and A3S6 showed the production of catechol and hydroxamate type siderophores in iron-restricted conditions in CAA medium. It is known that the siderophores produced by bacteria bind to the unavailable form of Fe^{3+} and make iron available to the plants, leading thereby to an increase in plant growth [39].

Production of plant cell wall degrading enzymes such as cellulase and pectinase was analyzed because this is an important mechanism for endophytic colonization [40]. Among the four

Table 2
Some key traits of Ni resistant endophytes.

Parameters	Endophytic bacterial strain			
	A3R3	A3S4	A2R6	A3S6
ACC deaminase, α-ketobutyrate mg^{-1} protein h^{-1} (nmol)	67.9 ± 6.2	40.0 ± 1.2	24.5 ± 1.4	nd
Phosphate solubilization (mg L^{-1})	138.2 ± 21.4	nd	nd	83.5 ± 11.1
IAA synthesis (mg L^{-1})	69.4 ± 3.2	53.0 ± 1.6	43.8 ± 1.6	60.8 ± 3.8
Catechol-type siderophore production (mg L^{-1})	83.3 ± 7.5	69.1 ± 3.9	nd	47.3 ± 2.1
Hydroxamate-type siderophore production (mg L^{-1})	60.5 ± 6.3	79.8 ± 3.6	nd	31.9 ± 2.1
Ni tolerance level (mg L^{-1})	750	750	1000	750

Average ± standard deviation from three samples. Nd: not detected.

strains tested, A3R3 displayed a positive cellulase activity, as indicated by the development of yellow-colored zone on NFB plates. However, all the four strains exhibited pectinase activity, which was expected considering the endophytic nature of the strains. Jha and Kumar [41] recently reported the existence of cell wall-hydrolyzing enzyme mediated endophytic colonization and suggested that cellulose and pectinase produced by *Klebsiella oxytoca* GR-3 might play an important role in plant–microbe interactions and intercellular colonization of root.

3.3. Screening of Ni resistant PGPE

In order to isolate an efficient PGPE, the Ni resistant PGPE (A3R3, A3S4, A2R6 and A3S6) were screened for their ability to promote the growth and Ni accumulation of *A. serpyllifolium* and *B. juncea* in Ni treated phytagar medium. It was interesting to find that none of the introduced Ni resistant PGPE showed any signs of pathogenicity towards *A. serpyllifolium* and *B. juncea*. In the absence of Ni, inoculation of PGPE induced an increase in fresh and dry weight of both *A. serpyllifolium* and *B. juncea* (Fig. 1). However, the maximum PGP effect was observed in A3R3. In the case of *A. serpyllifolium*, the strain A3R3 enhanced plant fresh weight and dry weight by 87% and 60%, respectively. Similarly, in *B. juncea* the strain A3R3 enhanced the fresh weight and dry weight by 162% and 177%, respectively. The application of Ni (5 mg Ni L^{-1}) to the phytagar medium did not affect the growth of *A. serpyllifolium*. However, *B. juncea* exposed to Ni demonstrated a significant ($p < 0.05$) reduction in plant growth. Reduction in the growth with application of Ni has been reported in various plant species [18,42]. Alteration of fundamental physiological/biochemical processes, e.g. leaf photosynthetic and transpiration activities [43], leaf chlorophyll content [44], have been attributed to excess Ni which could decrease fresh and dry matter yield. *A. serpyllifolium* inoculated with Ni resistant PGPE exhibited an increase in plant fresh and dry weight in the presence of Ni. However, the highest PGP effect was found for strain A3R3, which enhanced plant fresh and dry weight by 185% and 175%, respectively, compared with non-inoculated plants. Similarly the maximum PGP effect on Ni-treated *B. juncea* was observed after inoculation with strain A3R3. Metal resistant PGPE belonging to different genera such as *Pseudomonas*, *Microbacterium*, *Methylobacterium* and *Burkholderia* were found to have PGP characteristics that can potentially promote the plant growth and reduce metal stress symptoms in plants [10,11,45]. In addition to plant growth promotion, PGPE strain A3R3 significantly increased the Ni concentration in *A. serpyllifolium* and *B. juncea* by 36% and 20%, respectively (Fig. 2). Similar observations were also made by Zhang et al. [46]. The authors found that the inoculation of *Brassica napus* growing in 2.5 mg kg^{-1} of Cu-contaminated substrates with Cu resistant endophyte Pantoea agglomerans Jp3-3 significantly increased the concentration of Cu in above-ground tissues and roots by 31% and 78%, respectively, compared with respective non-inoculated control. The results clearly indicate that, among the four Ni resistant PGPE tested, the strain A3R3 was highly efficient at protecting both *A. serpyllifolium* and *B. juncea* from growth inhibition caused Ni and at enhancing the uptake of Ni by plants.

3.4. Influence of A3R3 on Ni toxicity in plants grown in Ni contaminated soil

Based on the promotion of plant growth and Ni accumulation of plants in phytagar assay, the strain A3R3 was selected for studying the effects of the strain on the plant growth and the uptake of Ni by *A. serpyllifolium* and *B. juncea* in soil. In the absence of Ni, inoculation of A3R3 did not greatly influence the growth of *A. serpyllifolium* (Fig. 3a and b) Fig. 3. However, *B. juncea* inoculated with A3R3 demonstrated a significant ($p < 0.05$) increase in plant

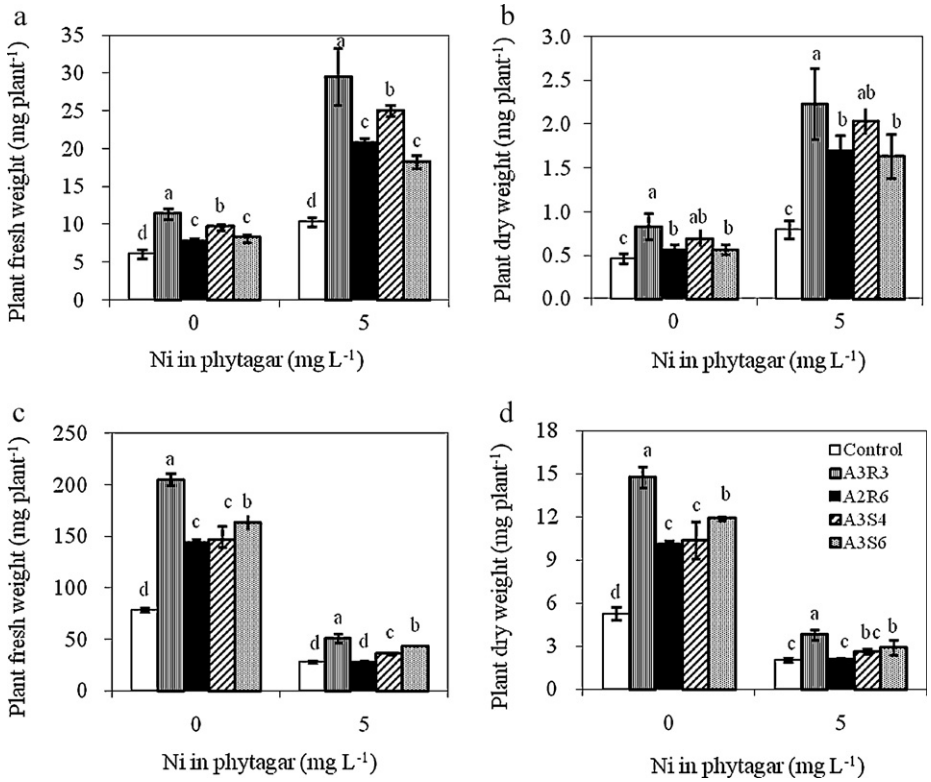

Fig. 1. Influence of Ni resistant PGPE on the fresh weight and dry weight of *A. serpyllifolium* (a, b) and *B. juncea* (c, d) grown in Ni-amended phytagar. Each value is the mean of triplicates. Error bars represent standard deviation. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

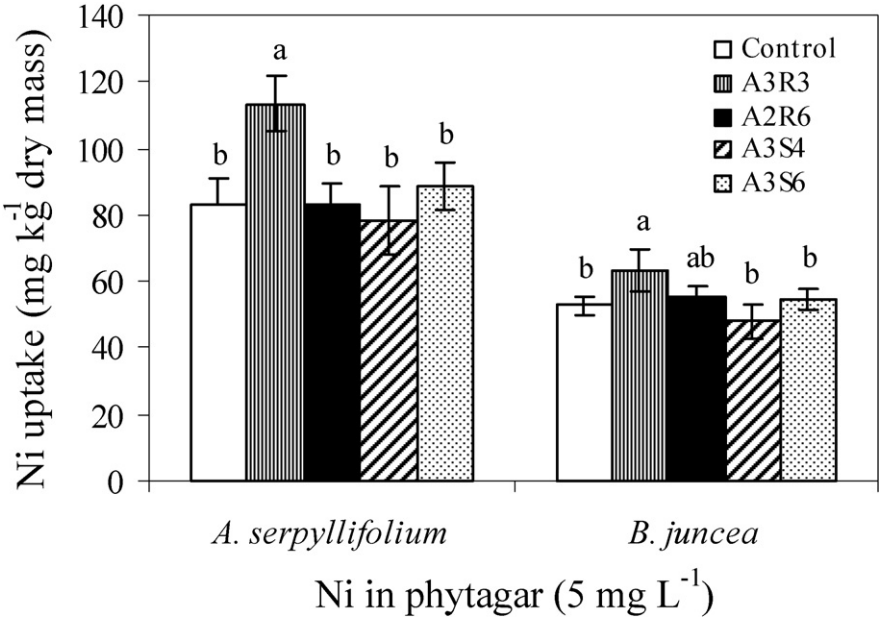

Fig. 2. Ni concentration in *A. serpyllifolium* and *B. juncea* grown in Ni-amended phytagar (5 mg L^{-1}). Each value is the mean of triplicates. Error bars represent standard deviation. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

fresh and dry weight (Fig. 3c and d). As shown in phytagar experiments, the application of Ni to the soil did not affect the growth of *A. serpyllifolium* negatively, but caused a significant increase in plant fresh and dry biomass. The beneficial effects of Ni on plant growth have already been reported in Ni hyperaccumulators, *Alyssum lesbiacum*, *A. bertolonii*, and Thlaspi goesingenses [47]. In contrast to *A. serpyllifolium*, the non-inoculated *B. juncea* exposed to different concentrations of Ni demonstrated a significant ($p < 0.05$) inhibition in plant growth. Panwar et al. [48] also reported similar results in *B. juncea* with increasing Ni content of soil (0–80 mg kg^{-1}). These results are not surprising, since serpentinophytes including *A. serpyllifolium* are generally understood as Ni hyperaccumulators that is well adapted to a high Ni concentration that *B. juncea* cannot tolerate [48,49].

A3R3 inoculations did not exhibit great influence on fresh and dry weight of *A. serpyllifolium* in the presence of Ni as compared with non-inoculated plants (Fig. 3a and b), whereas *B. juncea* inoculated with A3R3 exhibited a significant increase in plant fresh and dry weight in the presence of different concentrations of Ni. For instance, the strain A3R3 increased the fresh weight and dry weight of *B. juncea* by 50% and 45%, respectively, even at 450 mg Ni kg^{-1} soil, compared to non-inoculated but amended with the same dose

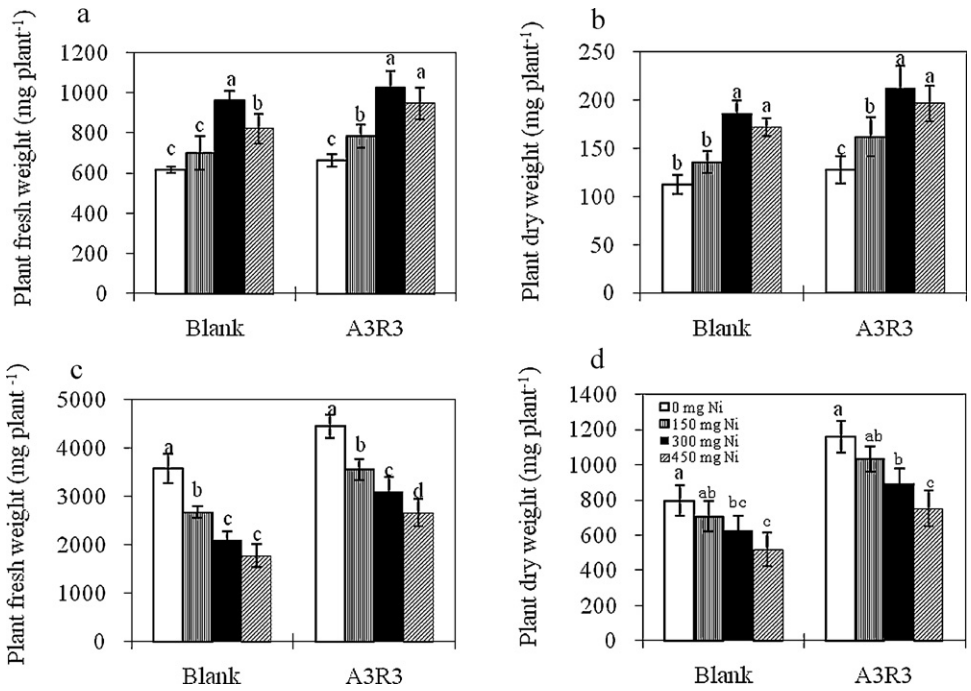

Fig. 3. Influence of Ni resistant PGPE A3R3 on the fresh weight and dry weight of *A. serpyllifolium* (a, b) and *B. juncea* (c, d) grown in Ni-amended soil. Each value is the mean of triplicates. Error bars represent standard deviation. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

Table 3
Colonization of strain A3R3 in the root and shoot interior of *A. serpyllifolium* and *B. juncea* (values in log CFU g^{-1} of shoot/root fresh weight).

Ni concentration (mg kg^{-1})	*A. serpyllifolium*		*B. juncea*	
	Shoot interior	Root interior	Shoot interior	Root interior
0	4.62 ± 0.02a	4.71 ± 0.02a	4.58 ± 0.05a	4.65 ± 0.08a
150	4.60 ± 0.02a	4.64 ± 0.01b	4.45 ± 0.04b	4.62 ± 0.09a
300	4.56 ± 0.01ab	4.55 ± 0.04c	4.27 ± 0.07c	4.44 ± 0.09b
450	4.50 ± 0.10b	4.38 ± 0.02d	4.19 ± 0.08c	4.30 ± 0.11b

Each value is the mean of triplicates. Error bars represent standard deviation. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

of Ni. This result is in agreement with a previous report describing increased biomass production of *Nicotiana tabacum* inoculated with Cd resistant endophytes and grown in Cd-supplemented soil [9]. In general, high concentrations of heavy metals in the rhizosphere soil interfere with the uptake of essential nutrients such as P, Fe and lead to plant nutrient deficiency and growth retardation [50]. Under such condition PGPE offer a biological rescue system capable of solubilizing/scavenging/mobilizing mineral nutrients (e.g. Fe, P, Zn) of soil and make them available to the plant roots during the initial colonization [37,51]. Further, ACC deaminase producing bacteria have been reported to reduce the deleterious effects of heavy metals on root elongation by reducing the stress ethylene level through hydrolytic cleavage of its precursor ACC [45,52]. In our study the observed benefits on the growth of *A. serpyllifolium* and *B. juncea* may be attributed to cumulative effects of A3R3, such as cleavage of ACC to α-ketobutyrate and ammonia by ACC deaminase, supply of Fe and P to the crop in addition to growth promoting substance IAA produced by this organism.

Although the PGPE possess several traits to promote the plant growth, colonization and survival in metal stress environment are very important factors for microbial assisted phytoremediation, as the activity of inoculated PGPE is necessary to produce beneficial substances. Hence, survival rate of the inoculated bacteria in plant tissue interiors was assessed. Though A3R3 originally isolated from the root tissues of *A. serpyllifolium*, it showed high level of colonization in shoot and root interior of both plants (Table 3). These observations strongly suggest that strain A3R3 is non-host specific colonizer and can move within tissues of the plant. Further, the endophytic nature of A3R3 is also evident from the presence of plant cell wall hydrolyzing enzymes, pectinase and cellulase by which the bacteria enter and colonize the plant tissues [53]. Further, the population density of A3R3 in tissue interior of *A. serpyllifolium* and *B. juncea* was not significantly affected in Ni (150 and 300 mg kg^{-1}) treated soil. However, at the highest concentration of Ni (450 mg kg^{-1}) a slight decrease in the population density was observed. Since most plant–microbe interactions are initiated at the level of colonization, the survival potential of the colonized microbes in metal stressed environment is likely to be closely linked to their metal resistance level. In addition to successful colonization in both host and non-host plants, the strain A3R3 was able to grow at increasing Ni concentrations (from 150 to 450 mg Ni L^{-1}) in liquid medium (data not shown) indicating an adaptation of A3R3 in Ni contaminated environment. Overall, our results indicate that A3R3 can form sustaining endophytic populations in the tissue interior of *A. serpyllifolium* and *B. juncea* and exhibit beneficial effects on plants irrespective of Ni stress.

Fig. 4 shows the Ni distribution profile in shoot and root systems of *A. serpyllifolium* and *B. juncea* grown at varying concentrations

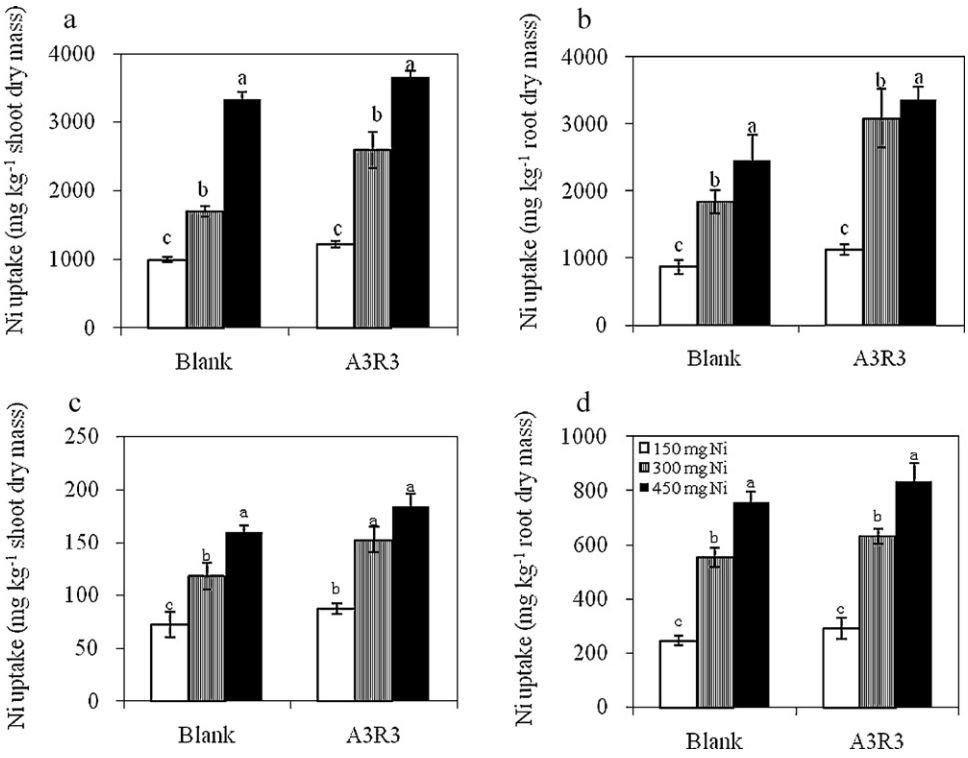

Fig. 4. Ni concentration in shoot and root of *A. serpyllifolium* (a, b) and *B. juncea* (c, d) grown in Ni-amended soil. Each value is the mean of triplicates. Error bars represent standard deviation. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

of Ni in the soil. Accumulation of Ni in the root and shoot systems increased with increase in the initial concentration of Ni in soil. However, *A. serpyllifolium* accumulated more Ni in both the shoot and root tissues compared with *B. juncea*. Further, the inoculation of the strain A3R3 significantly increased the accumulation of Ni in the tissues of *A. serpyllifolium* and *B. juncea* compared with respective non-inoculated controls. For instance, strain A3R3 increased the Ni concentration in the shoot tissues of *A. serpyllifolium* and *B. juncea* by 10% and 15%, respectively, when plants were grown in soil amended with 450 mg Ni kg^{-1} compared to respective non-inoculated plants. Recently, Sheng et al. [10] reported that inoculation of *B. napus* with PGPE *P. fluorescens* significantly increased the plant uptake of Pb when compared with the dead bacterial-inoculation control. They attributed this effect to the ability of PGPE to produce siderophores and to reduce soil pH. Similarly, Zaidi et al. [54] have also reported that microbial solubilizing inorganic phosphates facilitate the uptake of the metals from soil. In our study, the significant increase in Ni accumulation of plants caused by A3R3 could be attributed to the production of siderophore and the solubilization of P.

Analysis of the 1224 bp 16S rDNA sequence of the strain A3R3 using the BLASTn program at NCBI showed 100% sequence homology to *Pseudomonas* sp. Based on the biochemical features (data not shown) and 16S rDNA sequence analysis, the isolate A3R3 was identified as a strain of *Pseudomonas* sp. The sequences obtained have been deposited in NCBI databases under the accession number GU550663.

4. Conclusion

Modification of plants to obtain organisms with improved phytoremediation capabilities is generally carried out by integrating foreign DNA into plant genomes to produce transgenic plants [55]. Although the genetic manipulation may be a promising approach, these methods are expensive, time consuming and dependent on specific plant being studied. As an alternative approach, PGPE have been used to improve the phytoremediation efficiency without requiring integration of foreign DNA into the plant genome. Our study demonstrated that the inoculation of metal resistant PGPE, *Pseudomonas* sp. A3R3 seemed to be effective in promoting the phytoremediation potential of both host (*A. serpyllifolium*) and non-host (*B. juncea*) plants by improving either the Ni accumulation or biomass production. Although PGPE *Pseudomonas* sp. A3R3 might have significant potential to colonize tissue interior of host and non-host plants, not every PGPE possesses the ability to colonize multiple plants, and not every plants that has the potential to harbour several PGPE. Hence, further studies, including the role of *Pseudomonas* sp. A3R3 on the growth and phytoremediation potential of various hyperaccumulator plants in metal contaminated soils are under progress in order to test the usefulness of this novel isolate for future phytoremediation application.

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