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Effect of a Nickel-Tolerant ACC Deaminase-Producing *Pseudomonas* Strain on Growth of Nontransformed and Transgenic Canola Plants

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Abstract Four bacterial strains were isolated from soils at nickel-contaminated sites based on their ability to utilize 1-aminocyclopropane-1-carboxylate (ACC) as a sole source of nitrogen. The four isolates were all identified as *Pseudomonas putida* Biovar B, and subsequent testing revealed that they all exhibited traits previously associated with plant growth promotion (i.e., indoleacetic acid and siderophore production and ACC deaminase activity). These four strains were also tolerant of nickel concentrations of up to 13.2 mM in the culture medium. The strain, HS-2, selected for further characterization, was used in pot experiments to inoculate both nontransformed and transgenic canola plants (expressing a bacterial ACC deaminase gene in its roots). Plants inoculated with the HS-2 strain produced an increase in plant biomass as well as in nickel (Ni) uptake by shoots and roots. The results suggest that this strain is a potential candidate to be used as an inoculant in both phytoremediation protocols and in plant growth promotion.

Introduction

The remediation of metal-contaminated soils often involves excavation and removal of soil to secured landfills, a technology that is expensive and requires site restoration. However, in the past few years scientists have begun to explore the possibility of using plants to remove metal contaminants i.e., phytoremediation [8, 25]. A number of plants that can naturally accumulate large amounts of metal have been identified and proposed for use in the phytoremediation of metals. Unfortunately, the presence of very high concentrations of metals is often inhibitory to the growth of plants, even metal hyperaccumulating plants [15].

Plant growth-promoting bacteria from diverse origins can improve the growth of plants under stressful conditions [2, 16, 26] including the presence of high metal concentrations [5, 6, 9, 27]. In particular, 1-aminocyclopropane-1-carboxylate (ACC) deaminase-producing bacteria play an important role in the alleviation of different types of stress in plants, including the effect of heavy metals [13, 16].

The presence of a large number and wide range of nickel-resistant bacterial strains in nickel- and other metal-contaminated soils has been well documented [18, 21, 28]. A variety of mechanisms leads to nickel resistance in bacteria, including active membrane efflux systems, sequestration, and intracellular accumulation [4, 34].

In this study, bacterial strains were isolated from nickel-contaminated soil samples and the superior selected strain was used to examine its effect on the growth and nickel accumulation of nontransformed and transgenic canola (carrying a bacterial ACC deaminase gene expressed under the control of a root specific promoter) plants grown in nickel-contaminated soil was monitored. Strains that are plant growth promoters and are tolerant of high metal concentrations in the soil are likely to be important for the development of metal phytoremediation protocols. However, before these bacteria can be used in the field, it is essential that they demonstrate the desired traits under laboratory conditions.

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Materials and Methods

Isolation of Bacterial Strains

Pseudomonas putida UW4 [14, 17] was used as a control strain in some experiments. For the isolation of nickel-tolerant strains, soil samples from 2- to 10-cm depth were randomly taken from a nickel-contaminated field site in Port Colborne, Ontario, Canada. The soil consisted of 48.2% clay, 42.2% silt, and 9.6% sand; it contained 50–52% organic matter, 0.55–0.7% inorganic carbon, and 35–40.3% organic carbon, on a dry weight basis. The content of Ni was 6.3 and 2.9 μg Ni/g dry weight in the surface and sub-surface soil, respectively. Bacterial strains were isolated by the method of Penrose and Glick [24]. One-gram aliquots of plant rhizosphere soil were inoculated in 50 ml nutrient broth medium (3 g beef extract and 5 g peptone per liter) containing 0.5 μg/ml cycloheximide (to prevent the growth of fungi) at 200 rpm for 24 h at 30°C. One milliliter of each day-old bacterial culture was transferred to 50 ml of Dworkin and Foster (DF) minimal medium [10] with 0.5 μg/ml cycloheximide and incubated at 30°C for 24 h with 200 rpm shaking before a 1-ml aliquot was removed and used to inoculate 50 ml of DF medium with 300 mM ACC as the nitrogen source. After growth at 200 rpm for 24 h at 30°C, 1 ml of each culture was centrifuged, washed, resuspended in a 0.85% NaCl, plated onto solid DF minimal medium plus 300 mM ACC, and then incubated at 30°C for 72 h. Four colonies were selected (from different soil samples) and characterized by DNA sequence analysis of 16S rDNA and by use of a Biolog Kit (Biolog, Inc., Hayward, CA).

Strain Characterization

ACC deaminase activity and indoleacetic acid (IAA) were determined as described [23, 24]; siderophores were measured every 4 h during 48 h of cultivation [31]. An estimate of the tolerance of the bacterial strains to nickel was assessed by culturing the strains in the presence of 0, 2.3, or 13.2 mM of Ni (i.e., designated as zero, moderate, and high levels of nickel). MO minimal medium [29] containing tricalcium phosphate as the only P source was used for phosphate solubilization assays on plates [30].

To determine minimal inhibitory concentrations (MIC) for different antibiotics, i.e., ampicillin (β-lactam), kanamycin (aminoglycoside), novobiocin (quinolone), tetracycline (tetracycline), and erythromycin (macrolide), concentrations of antibiotic ranged from 0.125 to 512 μg/ml. After incubation for 24 or 48 h at 30°C with shaking, the OD_{600} was recorded.

Nickel Analysis

To ~30 mg of ground plant tissue (in an EDTA-rinsed porcelain mortar and pestle), 750 μl of 1 N HNO_3 was added and the mixture incubated for 3 h at 65°C. Digests were cooled to room temperature then centrifuged for 10 min at 20,000 *g*. Then 100 μl of each digest was added to 900 μl of Milli-Q water. Samples were analyzed for Ni content via graphite furnace atomic absorption spectroscopy (GF-AAS) using a Varian AA800 spectrophotometer with a GTA 100 graphite furnace provided by Dr. R. Playle of Wilfrid Laurier University, Waterloo, Ontario, Canada [6].

Total Ni content of soil samples was determined using nitric and hydrochloric acid digests of dry soil samples, and Ni measurements were made via inductively coupled plasma spectroscopy (ICP) by Laboratory Services, University of Guelph, Guelph, Ontario, Canada.

Plants

This work was performed using either nontransformed canola (*Brassica napus* var.Westar) or canola transformed with the ACC deaminase gene from *Pseudomonas putida* UW4 [32, 33]. The transformed canola line has two copies of the ACC deaminase gene under the control of the root-specific *Agrobacterium rhizogenes rolD* promoter [11]. This homozygous line was created through *Agrobacterium tumefaciens* transformation of canola callus culture.

Pot Experiments

These experiments were performed using subsurface soil, approximately >15 cm deep, from the same site where the strains were isolated, which contained 2.9 mg Ni/g soil, dry weight . Samples from a noncontaminated site, analyzed for comparison, contain 0.056 μg Ni/g soil, dry weight. The total number of culturable bacteria in the subsurface soil was 1×10^9 CFU/ml, while the number of ACC-utilizing bacteria was 2×10^7 CFU/ ml.

Seeds were surface sterilized, then inoculated with strain HS-2 at an OD_{600} of 0.5 [24]. One canola seed was planted per pot (55 mm square and 75 mm deep), with eight pots per treatment, and grown in a greenhouse with supplemental lighting for a 16-h photoperiod, with a daytime maximum of 25°C and a nighttime minimum of 20°C. Pots were watered as needed. Plants were harvested after 28 days. The entire experiment was repeated three times.

Statistics

Plant growth measurements and Ni concentrations are expressed as mean ± standard error of the mean (SE) for each treatment or group. Significant differences between treatments or groups were determined using analysis of variance followed by a Tukey test. All statistical analyses were conducted at $\alpha = 0.05$ using SYSTAT 10 (Statistical Package for the Social Sciences, 2000).

Results

Following the isolation of ACC-utilizing strains from nickel-contaminated soil, four different morphotypes were selected; all were identified as *Pseudomonas putida* Biovar B. Three of the four strains were fluorescent in King's B medium [20] after irradiation with 254-nm UV light (i.e., all but FT-3). Three of these strains grew to a much greater extent, following 24 h in 13.2 mM Ni, than either the control strain *P. putida* UW4 or isolate FT-3. A summary of the assayed traits for the four new isolates is presented in Table 1. Strain HS-2, which combined the highest nickel tolerance, the highest IAA and siderophore production, and a moderate level of ACC deaminase activity, was selected for further study. *P. putida* HS-2 was susceptible to kanamycin (MIC = 6 μg/ml) and tetracycline (MIC = 12 μg/ml) but resistant to ampicillin (MIC > 256 μg/ml), novobiocin (MIC > 256 μg/ml), and erythromycin (MIC = 96 μg/ml). Although antibiotic sensitivity is not involved in bacterial plant growth promotion, there is significant concern regarding the deliberate release of organisms that might increase the prevalence of antibiotic resistance in the environment. This strain could also solubilize mineral phosphate (data not shown).

To assess the ability of *P. putida* HS-2 to ameliorate the effects of nickel on canola, a 2^2 factorial design was used, with the plant (nontransformed or transgenic) and the inoculation with HS-2 as the variables [22, 29]. The percent emergence of canola seeds under these four treatment regimens is reported in Table 2. The presence of the ACC deaminase gene in the transgenic plant as well as the inoculation with *P. putida* HS-2 improved the percentage emergence of the plants (measured at either 7 or 11 days) in the presence of the high nickel concentration (2.9 mg Ni/g dry soil) of this soil.

Table 3 presents the values for different physiological responses of 28-day-old plants grown in the presence of 2.9 mg Ni/g dry soil. Shoot length is affected by both the inoculum and the type of plant. Shoot length is significantly greater following inoculation with *P. putida* HS-2, and slightly lower with the ACC deaminase transgenic plant compared to the nontransformed plant. For all of the measured parameters, inoculation with *P. putida* HS-2 always exerted a positive effect, enhancing plant growth and, also, Ni uptake by both shoots and roots. On the other hand, for these traits, there was no significant difference between nontransformed and transgenic plants.

Table 2 Percentage emergence of canola seeds planted in nickel-contaminated soil

Treatment		Time (days)	
Plant	Inoculum	7	11
Nontransformed	No bacteria	25	25
Transgenic	No bacteria	50	62.5
Nontransformed	*P. putida* HS-2	87.5	100
Transgenic	*P. putida* HS-2	62.5	100

Note: Each data point represents 16 seeds. The entire experiment was repeated three times

Discussion

ACC deaminase activity has a significant effect on plant growth promotion, in particular, facilitating plant growth in the presence of different types of stresses [16]. ACC deaminase production was previously found to be associated with nickel tolerance in a *Kluyvera ascorbata* strain [5, 6], and this trait was also associated with endophytic bacteria living within the roots of the nickel-hyperaccumulating plant *Thlaspi goesingense* in metal-contaminated soils [18].

In the experiments reported here, about 0.2% of the total culturable bacteria was able to grow with ACC as the sole source of nitrogen. The four different morphotypes isolated from the nickel-contaminated soil were all identified as *Pseudomonas putida* Biovar B. This is consistent with previous reports of a high proportion of pseudomonads among nickel-resistant bacterial communities [21]. The occurrence of nickel resistance as a relatively high-frequency trait in species of this genus, in particular, in *P. putida*, has been noted previously [7, 19].

The strain, *P. putida* HS-2, was selected for further studies due to its remarkable resistance to nickel, much higher than that reported previously for a *Kluyvera ascorbata* strain [5] or for a variety of rhizosphere isolates [18]. Inoculation of nontransformed as well as transgenic canola with *P. putida* HS-2 in the presence of nickel produced a significant enhancement of canola growth (shoot length, shoot and root dry weight) and, also, enhanced the uptake of nickel by the plant in both shoots and roots. Inoculation with the bacterium produced an increase in nickel content of 89% and 107% in shoots and roots,

Table 1 Traits of the four different bacterial isolates: mean ± SE

Strain	ACC deaminase activity[a]	Siderophores (mM DFA · A_{630}^{-1})[b]	IAA (μg · ml^{-1} · A_{630}^{-1})	Growth + 13.2 mM Ni (%)[c]
FT-1	2.90 ± 0.20	19.9 ± 2.3	2.4 ± 0.7	49.8 ± 0.7
HS-2	1.90 ± 0.10	17.4 ± 2.0	3.3 ± 0.4	64.8 ± 1.1
FT-3	3.47 ± 0.03	2.0 ± 0.2	1.5 ± 0.6	7.5 ± 0.5
FT-4	1.10 ± 0.10	3.5 ± 1.4	2.3 ± 0.4	52.8 ± 0.9

[a] μM α-ketobutyrate/mg protein/h

[b] DFA, desferal mesylate

[c] Percentage of the growth minus nickel

Table 3 Effect of *P. putida* HS-2 on the growth of canola (nontransformed or transgenic) in the presence of nickel after 28 days of growth: mean ± SE

Treatment		Shoot length (mm)	Shoot weight (mg)	Root weight (mg)	Ni in shoots (μg/g)	Ni in roots (μg/g)
Plant	Inoculum					
Nontransformed	No bacteria	12.2 ± 0.7	163 ± 3	16.7 ± 1	5.33 ± 0.4	2.9 ± 0.5
Transgenic	No bacteria	11.6 ± 0.9	210 ± 70	20 ± 9	6.3 ± 1.7	2.9 ± 1.3
Nontransformed	*P. putida* HS-2	16.1 ± 1.2	290 ± 40	37 ± 5	11.0 ± 0.9	6.1 ± 1.1
Transgenic	*P. putida* HS-2	13.0 ± 0.3	268 ± 32	37 ± 8	14.2 ± 1.4	5.2 ± 0.9

respectively, with nontransformed canola, while the increases in nickel content were 112% and 51% with the transgenic canola. These results compare favorably with the reported nickel uptake increases of from 17% to 32% when *Alyssum murale* plants were inoculated with a strain of *Microbacterium arabinogalactanolyticum* [1]. Whiting et al. [36] also reported metal-resistant bacteria isolated from the plant rhizosphere to be involved in increasing the availability of metals to plants.

Heavy metals cause stress in plants by inducing ethylene production [35], which can inhibit plant growth. The effect of bacterial ACC deaminase in lowering ethylene level in plants has been well documented [3, 5, 16]. In particular, a decrease in ethylene levels in canola plants grown in nickel-contaminated soil and inoculated with *K. ascorbata* was observed [5].

Interestingly, there was no effect of the type of plant (nontransformed or transgenic) on either metal accumulation or any of the measured growth parameters, except for shoot length. This suggests that promotion of plant growth in the presence of nickel by *P. putida* HS-2 depends on other factors (such as auxin or siderophores) in addition to ACC deaminase activity. For example, a siderophore-overproducing mutant of *K. ascorbata* was found to promote plant growth to a significantly greater extent than the wild type in the presence of inhibitory levels of heavy metals [6]. In addition, transgenic canola plants expressing an ACC deaminase gene in their roots accumulate approximately the same amount of nickel in shoots in comparison with nontransformed plants; however, the transgenic plants grow to a much greater size than the nontransformed plants [33]. Importantly, the nickel concentration in the experiments reported here was much higher than the level used in Ref. 33.

In agreement with the laboratory experiments reported here, in field experiments the addition of *P. putida* HS-2 significantly increased plant growth of nontransformed canola in Ni-contaminated soil [12]. In addition, in the field, *P. putida* HS-2 had no effect on plant growth of transgenic canola compared to nontransformed canola [12]. Since *P. putida* HS-2 is a natural, nonpathogenic bacterial isolate from nickel-contaminated soil, this bacterium may be an ideal adjunct to a phytoremediation strategy designed to remove nickel from the soil.

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