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Exploring the Plant Growth-Promotion Properties of Rhizospheric and Endophytic Bacteria Associated with *Robinia pseudoacacia* L. in Serpentine Soil

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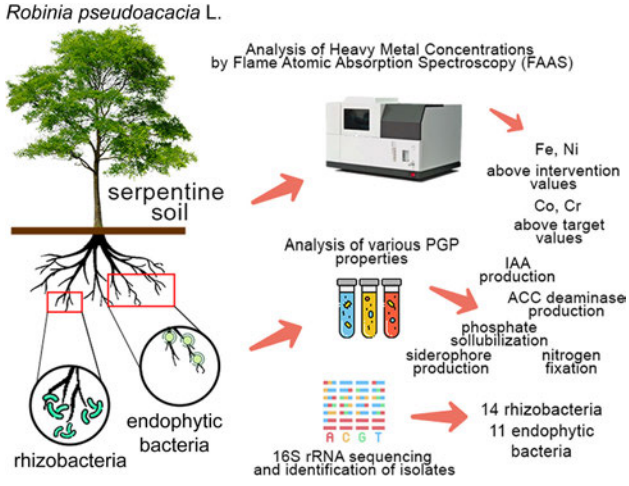
Abstract

Serpentine soils are characterized as a unique environment with low nutrient availability and high heavy metal concentrations, often hostile to many plant species. Even though these unfavorable conditions hinder the growth of various plants, particular vegetation with different adaptive mechanisms thrives undisturbed. One of the main contributors to serpentine adaptation represents serpentine bacteria with plant growth-promoting properties that assemble delicate interactions with serpentine plants. *Robinia pseudoacacia* L. is an invasive but adaptive species with phytoremediation potential and demonstrates extraordinary success in this environment. To explore more in-depth the role of plant growth-promoting serpentine bacteria, we isolated them and tested their various plant growth-promoting traits both from the rhizosphere and roots of *R. pseudoacacia*. Based on the demonstrated plant growth-promoting traits such as siderophore production, phosphate solubilization, nitrogen fixation, indole-3-acetic acid production, and ACC deaminase production, we sequenced overall 25 isolates, 14 from the rhizosphere and 11 from the roots. Although more efficient in exhibiting plant growth-promoting traits, rhizospheric bacteria showed a low rate of diversity in comparison to endophytic bacteria. The majority of the isolates from the rhizosphere belong to *Pseudomonas*, while isolates from the roots exhibited higher diversity with genera *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Lysinibacillus* and *Brevibacterium*/*Peribacillus*/*Bacillus*. The capacity of the described bacteria to produce siderophores, solubilize phosphate, and fix nitrogen highlights their central role in enhancing nutrient availability and facilitating *R. pseudoacacia* adaptation to serpentine soils. The findings highlight the potential significance of serpentine bacteria, particularly *Pseudomonas*, in contributing to the resilience and growth of *R. pseudoacacia* in serpentine environments.



K e y w o r d s: serpentine bacteria, *Robinia pseudoacacia* L., plant growth-promoting bacteria, heavy metals, 16S rRNA gene sequencing

Introduction

As firmly rooted life forms, plants concurrently cope with the adverse array of biotic and abiotic stress factors. They developed an intricate set of adaptive mechanisms to survive and thrive, altering their biochemical and genetic activity and adjusting physical barriers (Hu et al. 2018; Iqbal et al. 2021). In addition, many plant species improve their defense mechanisms by forming discrete interactions with beneficial microorganisms

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from soil (Trivedi et al. 2020). A particularly significant group of microorganisms for favorable interactions are the plant growth-promoting bacteria (PGPB), which mainly colonize roots, their surface, and the rhizosphere (Pii et al. 2015). PGPB can enhance overall plant health and resistance to different biotic and abiotic stressors through various mechanisms such as phosphate solubilization (Alori et al. 2017), production of siderophores (Saha et al. 2016), indole acetic acid (IAA) (Duca et al. 2014), deaminase 1-aminocyclopropane-1-carboxylic acid (ACC) (Glick 2014) and nitrogen fixation (Pii et al. 2015; Goswami and Deka 2020).

In heavy metal-burdened soils with low nutrient availability, such as serpentine, these plant-microbe interactions are even more evident in a plant's stress response. Serpentine outcrops represent distinctive types of ecosystems with specific chemical characteristics. Serpentine soils generally have a low concentration of macronutrients, Ca:Mg ratio, and high concentrations of heavy metals, especially Ni (Brooks 1987). An unfavorable environment conditioned the growth of particular vegetation resistant to the abovementioned drawbacks. Due to the extreme mineral composition, several authors underline the importance of serpentine bacteria. In various studies, PGPB from serpentine outcrops showed resistance to high concentrations of heavy metals and/or enhanced plant resilience (Rajkumar et al. 2009; Ma et al. 2009; Fan et al. 2018).

Robinia pseudoacacia L. is an originally North American woody plant species from the *Fabaceae* family, which was introduced in Europe a few centuries ago (Kolbek et al. 2004; Vítková et al. 2017). As a pioneer species, it inhabits many habitats, such as sandy and rocky outcrops, dry forests, alluvial habitats, and farm fields (Chytrý et al. 2008). Although it is considered an invasive species, the negative influence on the native plant communities is unclear. Many findings indicate clear homogenization and reduced herbaceous diversity, especially in the understory area (Peloquin and Heibert 1999; Benesperi et al. 2012). However, the results of a few other studies did not reveal any significant invasive properties of *R. pseudoacacia* (Von Holle et al. 2006; Sitzia et al. 2012). Despite its invasiveness, *R. pseudoacacia* is still used for the afforestation of anthropogenically-degraded habitats (Yüksek and Yüksek 2011; Hu et al. 2021). It is a promising alternative as a phytoremediation technique due to its large biomass and the potential to thrive in heavy metal-contaminated areas (Vlachodimos et al. 2013).

Other than the physical advantages (ways of dispersal, root system, and fast growth) the overall mechanisms of *R. pseudoacacia* resilience are not fully covered. In this study, we shed light on serpentine bacterial isolates with plant growth-promoting characteristics both from the rhizosphere and roots as an essential puzzle in an *R. pseudoacacia* success. To achieve this, we isolated bacterial strains from both the rhizosphere and roots of *R. pseudoacacia* and evaluated their plant growth-promoting (PGP) traits. These properties included siderophore production, phosphate solubilization, nitrogen fixation, indole-3-acetic acid (IAA) production, and ACC deaminase activity-essential functions that are known to enhance plant growth and stress tolerance in nutrient-poor and heavy-metal environments. We sequenced 25 isolates, comprising 14 from the rhizosphere and 11 from the roots, to further characterize their diversity. This study provides insight into the functional diversity of serpentine-associated PGPB and their potential engagement in *R. pseudoacacia* resilience in harsh habitats.

Experimental

Materials and Methods

Soil sampling. The soil sample was collected from the serpentine outcrop at Donja Paklenica, Bosnia and Herzegovina. From 10 cm depth, we sampled approximately 100 g of A-horizon or topsoil layer of undisturbed plot. Large and small soil particles were mixed and pooled as an individual sample. Samples were collected in triplicate using sterile bags and stored in an opaque portable refrigerator. For the isolation of bacteria, approximately 100 g of bulk soil was collected in the proximity of *R. pseudoacacia* roots. The sample was taken in triplicate, placed in sterile bags, and stored in a transportable refrigerator.

Extraction of heavy metals and macroelements from soil sample. To remove moisture, soil particles were air-dried on a clean surface in a thin layer. After removing surface debris, heavy metals and macroelements were extracted following ISO 11466:1995 (1995). Briefly, 3 g of soil sample was mixed in 28 ml of aqua regia (21 ml HCl, 7 ml HNO_3) and heated until boiled. The mixture was preserved for 16 h at 20°C and then boiled using a reflux condenser. After cooling, the suspension was filtered through a filter paper and diluted with distilled water up to a final volume of 100 ml. After adjusting the volume, the concentration of the soil was 30 mg/ml, which was utilized as an input in flame atomic absorption spectroscopy (FAAS) (AA-7000; Shimadzu Corporation, Japan) to express elements in mg/kg.

Analysis of heavy metal and macroelement concentrations by flame atomic absorption spectroscopy (FAAS). A series of working solutions and standards were prepared using certified standard stocks (Merck KGaA, Germany). According to ISO 11047:1998 (1998), the concentrations of Ca, Mg, K, Cd, Cr, Cu, Co, Fe, Mn, Ni, Pb, and Zn were specified using the atomic

absorption spectrophotometer AA-7000 (Shimadzu Corporation, Japan).

Assessment of soil contamination. Index of geoacummuation (Igeo) and enrichment factor (EF) were used to assess the presence of anthropogenic contamination in soil. EF is a reliable tool to determine the differences between naturally occurring and anthropogenic sources of pollutants (Morillo et al. 2002; Kumar et al. 2012). The EF approach requires normalizing one metal concentration to the reference element to differentiate natural from anthropogenic contaminants. Due to their stability in the soil, Al, Mn, Fe, and Rb are most frequently used as reference elements (Sinex and Wright 1988; Mucha et al. 2005; Lizarraga Mendiola et al. 2008). In our study, we used Fe as a reference element. To calculate the enrichment factor, we used the following equation:

$$EF = \frac{\left(C_{Metal}/C_{RE}\right)soil}{\left(C_{Metal}/C_{RE}\right)control}$$

C_{Metal} denotes a heavy metal concentration, whereas C_{RE} represents the reference heavy metal element concentration in soil and control samples (Okedeyi et al. 2014). The results were interpreted according to Sutherland's (Sutherland et al. 2000) scale for evaluating the types of contamination (Table I).

Table I
Enrichment factor (EF) scale, according to Sutherland (2000).

EF = < 2	deficient to low enrichment
EF = 2–5	moderate enrichment
EF = 5–20	considerable enrichment
EF = 20–40	high enrichment
E = > 40	extremely high enrichment

Igeo was employed for the evaluation of the soil contamination using the following equation (Müller 1979):

$$Igeo = log_2 \cdot \left(\frac{C_n}{1.5\ B_n}\right)$$

C_n represents the heavy metal concentration in the soil, whereas B_n represents the geochemical background concentration of the same element. To limit the influence of any potential variations in the background, we used 1.5 factor (Lizarraga Mendiola et al. 2008). The results of determined Igeo values were interpreted using contamination categories suggested by Huu et al. (2010) (Table II). The method was initially developed to assess the contamination of river bottom sediments, but it can also be used to evaluate soil pollution (Loska et al. 2004).

Table II
Contamination categories for Igeo values (Huu et al. 2010).

Igeo < 0	unpolluted
0 ≤ Igeo < 1	unpolluted to moderately contaminated
1 ≤ Igeo < 2	moderately contaminated
2 ≤ Igeo < 3	moderately to strongly contaminated
3 ≤ Igeo < 4	strongly contaminated
4 ≤ Igeo < 5	strongly to extremely contaminated
Igeo ≥ 5	extremely contaminated

Isolation and cultivation of bacteria from soil and roots. Rhizospheric bacteria were isolated from soil samples using a sterile 0.85% NaCl solution. The mixture of 1 g of soil and 100 ml of NaCl solution was vigorously shaken and left to stand still for large particles to settle. The supernatant was used to prepare a series of dilutions in 1/10, 1/100, and 1/1000 ratios. To isolate endophytic bacteria, roots were cleaned of soil particles and shortly surface sterilized for 10 min with 5% sodium hypochlorite (NaClO) and washed three times with sterile distilled H_2O afterward. Roots were chopped into smaller pieces (2–3 cm long) with a sterile scalpel and transferred to a Petri dish with sterile 0.85% NaCl. With occasional stirring, roots were left in the solution for 2–3 h, and then the solution was smeared on a solid nutrient medium. On a yeast mannitol agar (YMA) medium, we added between 100–200 µl of previously prepared solutions and incubated at 22–25°C for 24–48 h. After incubation, only distinct, morphologically differentiated and conspicuous colonies were selected and transferred to a tryptone yeast (TY) solid medium to obtain bacterial monocultures. Isolated rhizospheric bacteria are marked as Rb, while endophytic bacteria are marked as Eb. Overall, 25 isolates were selected for the PGP properties analysis, 14 Rb (Rb1–Rb14) and 11 Eb (Eb1–Eb11).

Heavy-metal resistance analysis. All identified PGPB were grown in the presence of Cu, Ni, and Co at 22°C. $CuSO_4 \cdot 5H_2O$ (100 mg/l and 200 mg/l), $NiSO_4 \cdot 6H_2O$ (100 mg/l and 200 mg/l), and $CoCl_2 \cdot 6H_2O$ (100 mg/l) were each added to the TY solid medium at the specified concentrations. Firstly, bacteria were incubated in TY broth medium with agitation of 130 rpm at 25°C and transferred onto solid TY medium. After 14 days of incubation at 22°C, observable growth was marked with the special character +, whereas the absence of growth was marked with –.

Qualitative analysis of siderophores. CAS assay was used to analyze siderophores qualitatively (Kumar et al. 2017). Briefly, in prepared King's B agar (20 g of peptone; 1.5 g K_2HPO_4; 1.5 g $MgSO_4 \cdot 7H_2O$; 10 ml glycerol and 15 g of agar; pH 7.2 ± 0.2) (King et al. 1954) we added 100 ml of CAS reagent (60.5 mg dissolved in 50 ml H_2O; 10 ml $FeCl_3 \cdot H_2O$ and 72.9 mg HDTMA

dissolved in 40 ml H_2O) (Schwyn and Neilan 1987). Selected isolates were incubated in TY broth solution at 25°C for 24 h and 130 rpm. The absorbance of culture was measured at 600 nm, and OD was set at 0.5. To assess siderophore production, we performed the well-diffusion test. In the perforated CAS agar wells (5–6 mm), 20 µl of each bacterial culture was added. The growth was monitored for 7 days, and the radius of orange zones around the bacterial colonies was expressed in mm afterwards.

Quantitative analysis of siderophores. Isolates were cultivated in the succinate broth medium without Fe (4 g sodium succinate; 1 g $(NH_4)_2SO_4$; 3 g K_2HPO_4 and 0.2 g $MgSO_4 \cdot 7H_2O$; pH 7 ± 0.2). After incubation for 48 h at 28°C and 120 rpm, bacterial cultures were centrifuged for 10 min at 5,000 rpm. The supernatant was mixed with CAS in a 1:1 ratio and measured using a microplate reader (Multiskan™ FC Microplate Photometer; Thermo Scientific™, Thermo Fisher Scientific, Inc., USA) at 630 nm. The succinate medium without Fe was used as a control (Senthilkumar et al. 2021). The percentage of siderophore units was calculated according to the following equation:

$$\frac{Ar - As}{Ar} \times 100$$

where Ar is the absorbance of the control sample and As represents the absorbance of the bacterial sample.

Qualitative analysis of phosphate solubilization. We employed the phosphate solubilization test by Ambrosini and Passaglia (2017) for the phosphate solubilization properties. After incubation for 24 h at 25°C in TY broth medium, the absorbance of the bacterial cultures was measured at 600 nm, and OD was set to 0.5. In glucose yeast (GY)/tricalcium phosphate agar solid medium (10 g glucose; 2 g yeast extract; 15 g of agar; pH 7.2 ± 0.2), we added 100 ml of bromophenol blue solution (0.025 g of bromophenol blue in 100 ml of distilled H_2O). After medium autoclavation, sterile K_2HPO_4 (50 ml) and 10% $CaCl_2$ (100 ml) were added. A well-diffusion test was employed to determine the phosphate solubilization properties. In the perforated GY/tricalcium phosphate agar wells (5–6 mm), 20 µl of each bacterial culture was added. The growth was monitored for 7 days, and the radius of clear zones around the bacterial colonies was expressed in mm afterwards.

Qualitative analysis of nitrogen fixation. Isolates were inoculated into semi-liquid medium without N_2 (5 g of malic acid; 4 g KOH; 0.5 g K_2HPO_4; 0.05 g $FeSO_4 \cdot 7H_2O$; 0.1 g $MnSO_4 \cdot 7H_20$; 0.02 g NaCl; 0.01 g $CaCl_2$; 0.0002 g Na_2MoO_4; 2 ml of ethanolic solution bromothymol blue as an indicator; 1.75 g agar; pH 6.6–7). After 7 days of incubation at 33 ± 2°C, isolates with nitrogen-fixation ability are characterized by white pellicles under the surface of the medium and the change of color from green to blue due to an increase in pH (Latt et al. 2018). Observable change of color was marked with the special character +, whereas the absence of color change was marked with −.

Quantitative analysis of indol-3-acid (IAA) production. Bacterial isolates were grown in TY broth medium amended with 1 mg/ml of tryptophane. After an incubation period of 96 h at 32 ± 2°C and 130 rpm, bacterial cultures were centrifuged at 5,000 rpm for 10 min. In a 1:1 ratio, Salkowski reagent (150 ml H_2SO_4; 250 ml ddH_2O; 7.5 ml 0.5 M $FeCl_3$) was mixed with grown cultures and measured at 530 nm on a spectrophotometer UVmini-1240 (Shimadzu Corporation, Japan). The concentration of produced IAA was determined according to the standard curve, which was produced by the series of IAA dilutions (10–200 µg/ml). The obtained values are expressed in µg IAA/ml (Goswami et al. 2015).

Qualitative analysis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase production. Before the initial sterilization and experiment, all laboratory materials were washed thrice in 6M HCl and distilled water afterward. The activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase was determined by the ability of bacteria to utilize ACC as a source of N_2. Isolates were grown in King's B broth medium for 48h at 28–30°C and 160–180 rpm. After the cultivation period, cultures were centrifuged at 5,000 rpm for 10 min. The supernatant was removed, and the bacterial pellet was resuspended in sterile 0.85% NaCl. This step was repeated three more times to remove leftovers of nutrition from King's B medium. DF agar solid plates (2 g of glucose; 2 g of sodium gluconate; 4 g K_2HPO_4; 7.5 g $Na_2HPO_4 \cdot 2H_2O$; 0,2 g $MgSO_4 \cdot 7H_2O$; 2 g of citric acid; 100 µl DF solution 1; 100 µl DF solution 2 and 20 g of agar; pH 7.2 ± 2°C) with and without added ACC were used for the inoculation of 2 µl bacterial suspension. After an incubation period of 7 days, differences in growth intensity were observed what indicates ACC deaminase activity (Ambrosini and Passaglia 2017). Observable growth was marked with the unique character +, whereas the absence of growth was marked with −.

16S rRNA sequencing and identification of isolates. For the identification of bacterial isolates with PGP properties, we used the 16S rRNA gene sequencing. Considering the ubiquity among bacterial species and the presence of highly conserved and variable regions, this gene was successfully employed in detection, identification, and classification. The PCR reaction was prepared according to the universal protocol established by Woese (1987). The following universal primers were employed for 16S rDNA: 16S-FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-RP2 (5'-ACGGCTACCTTGTTACGACTT-3'). The overall

volume of the PCR reaction was 50 µl and it contained 0.4 µl forward and reverse primer, 1 × RedTaq, 0.5 mM $MgCl_2$ and 1 µl of the sample (bacterial colonies lysate in distilled H_2O at 95°C for 15 min). The PCR reactions for 35 cycles were used according to the following thermal protocol: initial denaturation at 94°C for 10 min, denaturation at 94°C for 1 min, annealing at 45°C for 1.5 min, elongation at 94°C for 1.5 min, and final elongation at 72°C for 7 min., Gel electrophoresis was performed on 1.5% agarose gel for the validation of PCR products. In the further analysis, we included only PCR products with a specific band size of ≈ 1,500 bp. GenElute™ PCR Clean-Up (Sigma-Aldrich®, Merck KGaA, Germany) was used to purify PCR products. After measuring the concentrations of PCR products on Qubit® 2.0 Fluorometer (Invitrogen™, Thermo Fisher Scientific, Inc., USA) using HS (high sensitivity) Qubit assay, we prepared Sanger sequencing reactions (10–40 µl) using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, Thermo Fisher Scientific, Inc., USA). The overall volume of the PCR reaction was 20 µl containing 4 µl BigDye™ Terminator v3.1 Ready Reaction Mix, 2 µl BigDye™ Terminator v3.1 Sequencing buffer, 2 µl 3.2 µM forward primer, 3 µl H_2O, and 9 µl of product. The concentration of PCR products in reaction varied between 3–10 ng. Sequencing PCR reactions for 25 cycles were used according to the following BigDye™ Terminator v3.1 thermal protocol: initial denaturation at 96°C for 1 min, denaturation at 94°C for 10 sec, annealing at 50°C for 5 sec, elongation at 60°C for 4 min, and final store at 4°C. After the sequencing PCR reaction, samples were purified using SigmaSpin™ Sequencing Reaction Clean-Up (Sigma-Aldrich®, Merck KGaA, Germany). After drying the elution, we added 10 µl of formamide for sequencing (3500 Genetic Analyzer; Applied Biosystems™, Thermo Fisher Scientific, Inc., USA). Due to the significance of variable region 3 16S rDNA (V3), the amplicons were sequenced using a forward primer (5'-AGAGTTTGATCCTGGCTCAG-3'). DNA sequences were analyzed in Data Collection Software 3 on a 3500 Genetic Analyzer (Applied Biosystems™, Thermo Fisher Scientific, Inc., USA). BioEdit 7.7 software was employed to edit raw sequences. The obtained sequences were identified by comparing them with sequences deposited in NCBI GenBank (Sayers et al. 2020). For sequence similarity, we used BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1990), paying attention to two parameters: query size and sequence similarity.

Statistical analysis. All obtained concentrations for quantitative analysis are performed in triplicate and given as an average value in the results. We employed Student's *t*-test to compare Rb and Eb using PAST 4.10 (Hammer et al. 2001). Isolates that did not exhibit certain PGP characteristics were excluded from the particular statistical analysis. Even though we first tested the PGP traits of isolates and then performed 16S sequencing, for simplified interpretation and discussion of the results, we included the identified sequenced bacteria starting from the first interpretation of the presented PGP characteristic.

Results

Concentrations of heavy metals and macroelements in soil sample. The concentrations of heavy metals in soil are presented in descending order: Fe > Ni > Mn > Cr > Co > Zn > Cu > Cd. The concentration of Ni (1,912.45 mg/kg) in soil exceeded the target value (TV) and intervention value (IV) by more than ninefold. As predicted from serpentine soil, a high level of Fe (16,983 mg/kg) concentration was observed as well. Although not above IV values, Co (87.7 mg/kg) and Cr (203.42 mg/kg) also exceeded TV. The EF ladder revealed extremely high enrichment for Ni (56.305), while considerable enrichment was detected for Co (8.607) and Cr (5.208). Moderate enrichment was measured for Cu (2.904). Other heavy metals (HMs) except Fe and Cd (not calculated) showed deficient to low enrichment (Mn, Pb and Zn). Igeo index uncovered extreme contamination for Ni (5.202) and moderate to strong contamination for Co (2.634). Other HMs, except for Fe and Cd (not calculated), showed deficiency in low enrichment (Mn, Pb, and Zn). Igeo index uncovered extreme contamination for Ni (5.202) and moderate to strong contamination for Co (2.634). Other HMs, according to Igeo, exhibited moderate contamination (Cr and Cu), or were unpolluted (Fe, Zn, Pb) to moderately contaminated (Mn). The concentrations of heavy metals in soil, EF, and Igeo index are presented in Table III. The content of macroelements indicated low levels of Ca and K in the soil (Table IV). In contrast, high Mg and Fe content were observed. The Ca/Mg ratio was extremely low indicating severe imbalance in which Mg suppresses Ca uptake.

Heavy metal resistance. All Rb isolates exhibited growth on Ni 100 mg/l, Ni 200 mg/l, Cu 100 mg/l, and Co 100 mg/l except Rb9-*Bacillus* sp., which only grew on TY medium supplemented with Ni 100 mg/l. Eb isolates were generally more susceptible to the mentioned HM concentrations, with only four isolates on Cu 100 mg/l and Co 100 mg/l and two isolates on Ni 100 mg/l. HM resistance results are presented in Table V.

Qualitative analysis of nitrogen fixation. All selected isolates except Eb5 and Eb8 showed nitrogen fixation properties, which were observed in color change from green to blue and pellicle formation (Table V). Although Rb, compared to Eb isolates, reached the final change after 4 days, this qualitative

Table III
Heavy metal concentration in soil collected from Donja Paklenica.

Soil type		Cd	Ni	Mn	Pb	Co	Zn	Cr	Cu	Fe
Donja Paklenica	HM (ppm)	0	**1,912.45**	1042.51	5.76	**87.7**	33.69	**203.42**	22.19	**16,983**
	EF	–	56.305	1.637	0.242	8.607	0.749	5.208	2.904	–
	Igeo	–	5.202	0.238	−2.505	2.634	−0.891	1.9178	1.0570	−0.472
TV	HM (ppm)	0.8[a]	35[a]	–	85[a]	20[a]	50[a]	100[a]	36[a]	–
IV	HM (ppm)	12[a]	210[a]	3,000–40,000[c]	530[b]	240[b]	720[b]	380[a]	190[b]	3,000–500,000[d]

TV – target value, maximum of desirable levels in unpolluted soil;
IV – intervention values, serious concentration of heavy metals in soil, remediation needed.
Heavy metal concentrations above TV and IV values are marked in bold.
[a] – Denneman and Robberse 1990, [b] – VROM 93561/b//4-94 1221/027 (1994)
[c] – ATSDR, 2008 [d] – Eddy et al. 2006

Table IV
The content of macroelements in soil collected from Donja Paklenica.

Locality/Element	Ca (%)	Mg (%)	K (%)	Ca/Mg
Donja Paklenica	0.009	1.439	0.036	0.0063

Table V
All Identified Rb and Eb with heavy metal resistance, nitrogen fixation, and ACC deaminase activity.

Isolate	PGP properties		Heavy metal resistance				
	Nitrogen fixation	ACC deaminase activity	Growth on TY agar plates supplemented with heavy metals 14 d				
	Blue color, pellicle 7 d	Growth on DF + ACC agar plates	Ni 100 mg/l	Ni 200 mg/l	Cu 100 mg/l	Cu 200 mg/l	Co 100 mg/l
Rb1-*Pseudomonas* sp.	+	+	+	+	+	–	+
Rb2-*Pseudomonas* sp.	+	+	+	+	+	–	+
Rb3-*Pseudomonas* sp.	+	+	+	+	+	–	+
Rb4-*Pseudomonas* sp.	+	+	+	+	+	–	+
Rb5-*Pseudomonas* sp.	+	+	+	+	+	–	+
Rb6-*Pseudomonas* sp.	+	+	+	+	+	–	+
Rb7-*Pseudomonas* sp.	+	+	+	+	+	–	+
Rb8-*Pseudomonas* sp.	+	+	+	+	+	–	+
Rb9-*Bacillus* sp.	+	+	+	–	–	–	–
Rb10-*Pseudomonas* sp.	+	+	+	+	+	–	+
Rb11-*Pseudomonas* sp.	+	+	+	+	+	–	+
Rb12-*Pseudomonas* sp.	+	+	+	+	+	–	+
Rb13-*Pseudomonas* sp.	+	+	+	+	+	–	+
Rb14-*Pseudomonas* sp.	+	–	+	+	+	–	+
Eb1-*Bacillus* sp.	+	+	–	–	+	–	+
Eb2-*Staphylococcus* sp.	+	+	+	–	+	–	+
Eb3-*Bacillus* sp.	+	+	–	–	+	–	+
Eb4-*Bacillus* sp.	+	–	–	–	+	–	+
Eb5-*Bacillus* sp.	–	–	–	–	–	–	–
Eb6-*Bacillus* sp.	+	+	+	–	–	–	–
Eb7-*Pseudomonas* sp. Eb8-*Brevibacterium*/	+	–	–	–	–	–	–
Peribacillus/*Bacillus* sp.	–	+	+	–	–	–	–
Eb9-*Lysinibacillus* sp.	+	–	–	–	–	–	–
Eb10-*Bacillus* sp.	–	–	–	–	–	–	–
Eb11-*Bacillus* sp.	+	+	–	–	–	–	–

method did not provide us with a distinction between isolates within a group.

Qualitative analysis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase production. Nineteen isolates, 13 Rb and 6 Eb (Table V), produced ACC deaminase, which was detected throughout their ability to grow on DF + ACC agar plates.

Qualitative and quantitative analysis of siderophores. Statistical analysis of diameter size showed significant differences ($p < 0.0001$) between Rb and Eb isolates. After 7 days, the average values of diameter size for Rb and Eb were 13.29 mm and 5.5 mm, respectively. The largest diameter was observed by Rb9-*Bacillus* sp. (20 mm), while only Eb9-*Lysinibacillus* sp. did not show any activity regarding siderophore production using the qualitative method (Fig. 1). A significant difference ($p < 0.0004$) between Rb and Eb was observed in the quantitative analysis of the siderophore production rate as well (Fig. 2). The average percentage of siderophore units for Rb and Eb was 72.48% and 38.64%, respectively. The highest production showed Rb1-*Pseudomonas* sp. (96.70%), while the lowest was detected for Eb11-*Bacillus* sp. (11.46%).

Qualitative analysis of phosphate solubilization. Out of 25 selected isolates, 17 isolates showed phosphate solubilization activity. Between Rb and Eb isolates, there was no significant difference (Fig. 3). The average diameter size for Rb and Eb was 5.16 mm and 5.75 mm, respectively. The largest diameter of 8 mm was observed at Rb14-*Pseudomonas* sp. and 7 mm at Eb4-*Bacillus* sp.

Quantitative analysis of indol-3-acid (IAA) production. No statistical difference ($p < 0.73$) was observed between Rb and Eb isolates (Fig. 4). The highest concentration of IAA production in Rb with 614.66 µg/ml was detected in Rb13-*Pseudomonas* sp. Similarly, in the Eb group, a concentration of 699.89 µg/ml was observed in Eb2-*Staphylococcus* sp.

16S rRNA gene sequencing and identification of isolates. After editing with BioEdit software, the sequences were approximately 550 bp in length. The 16S rRNA gene sequencing of isolates clearly showed differences in genus diversity between Rb and Eb. All isolates from the rhizosphere except Rb9 (*Bacillus* sp.) belong to the *Pseudomonas* genus. On the other hand, endophytic isolates are more diverse and related to five different genera: *Bacillus*, *Pseudomonas*,

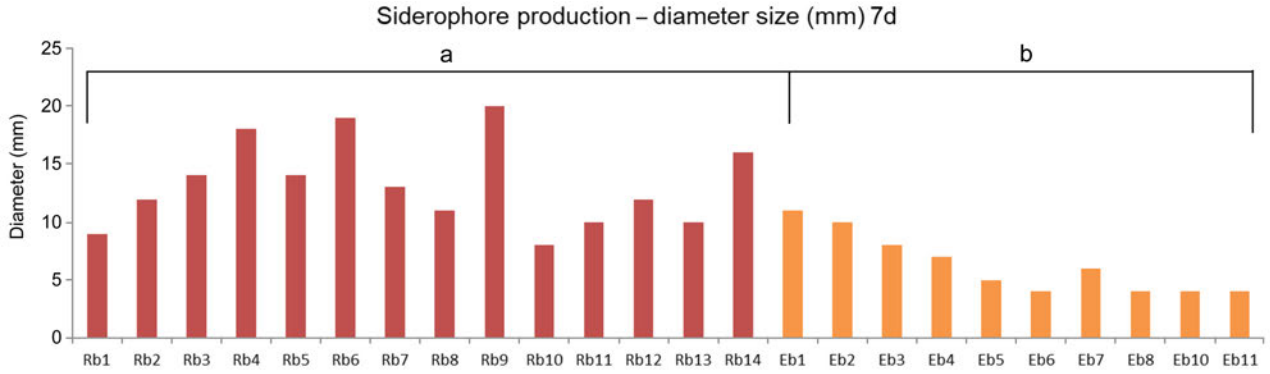

Fig 1. Qualitative analysis of siderophore production of Rb and Eb isolates. Different characters indicate statistical differences between groups ($p < 0.05$).

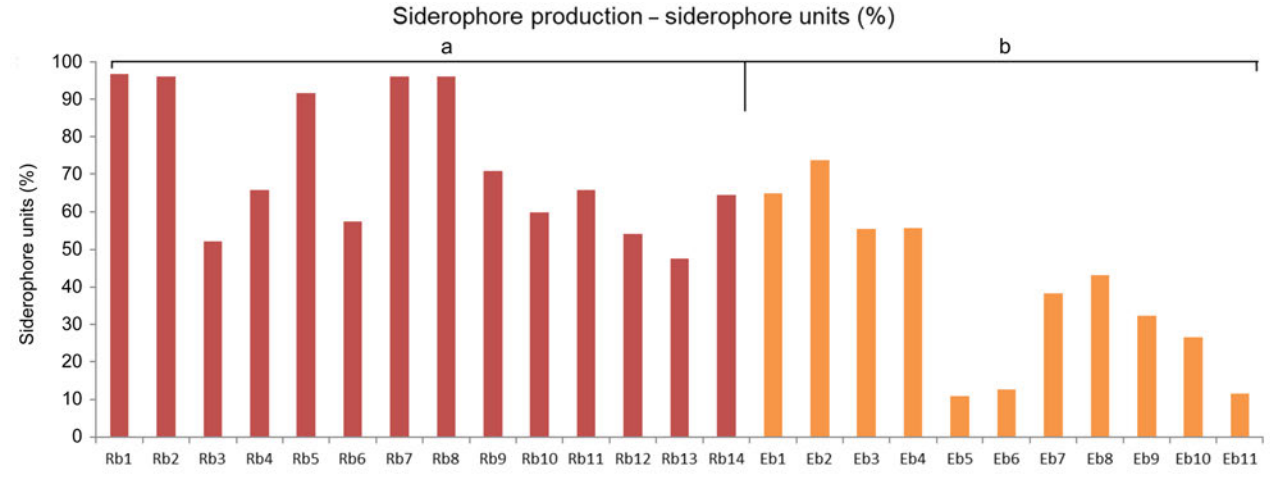

Fig 2. Quantitative analysis of siderophore production of Rb and Eb isolates. Different characters indicate statistical differences between groups ($p < 0.05$).

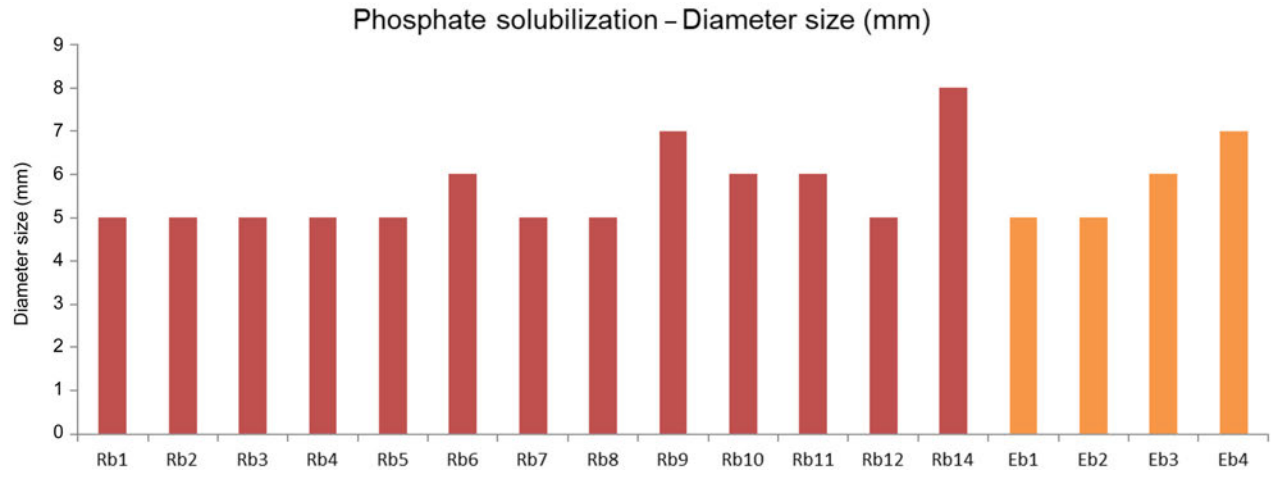

Fig 3. Qualitative analysis of phosphate solubilization of Rb and Eb isolates.

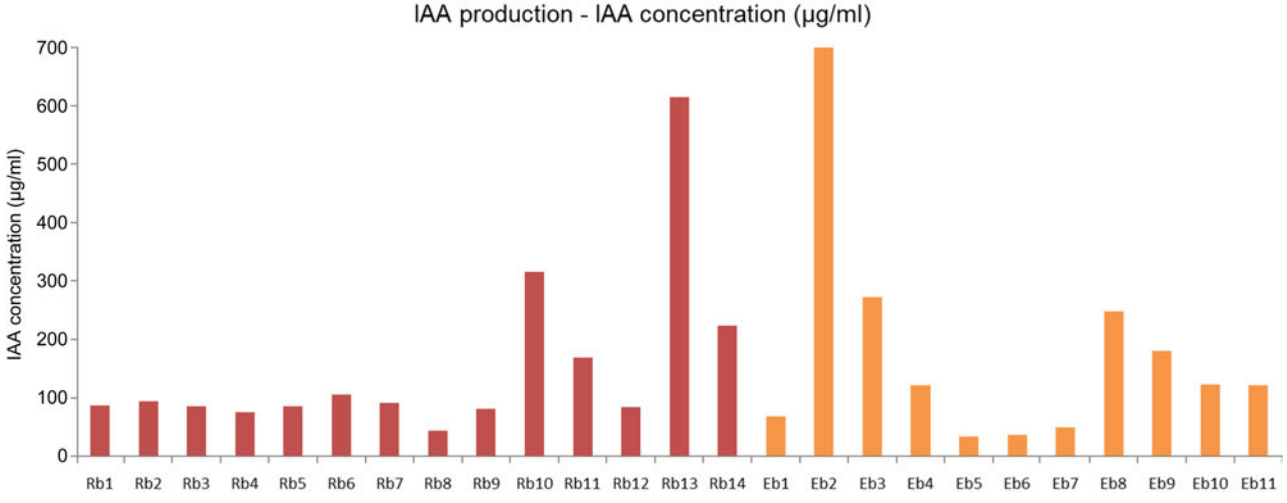

Fig 4. Quantitative analysis of indol-3-acid (IAA) production of Rb and Eb isolates.

Staphylococcus, *Lysinibacillus*, and *Brevibacterium*/*Peribacillus*. The results of BLAST results after sequence editing are presented in Table VI.

Discussion

With the extreme edaphic conditions and plant communities that differently influence soil properties, serpentines represent a unique complex for microbial studies. Even though soil chemistry is a crucial factor in plant survival, the effects of plant-microbe interactions contribute significantly to overall plant health (Kumar and Verma 2018). Statistical analysis of HMs in soil showed high concentrations of Ni, Fe, Co, and Cr that exceeded TV. These results were expected since serpentine soils are abundant with heavy metals such as Ni, Fe Co, and Cr (Kierczak et al. 2021). Serpentine soils are especially rich in Ni (Brooks 1987) and extreme soil contamination revealed by the Igeo index was anticipated.

In contrast, Cd and Pb are usually found in scarcity, which is also confirmed in our results. As serpentine soils are derived from ultramafic rocks, which contain ferromagnesian minerals susceptible to weathering, extremely high Mg and Fe concentrations were also expected. One of the essential hallmarks of serpentine soils is also the low concentration of Ca, K, and P. As the analyzed Ca concentration is well below the average concentration in soil, Ca/Mg ratio showed to be very low (0.0063). Loew and May (1901) were the first ones to conclude that the low ratio of these two elements is the main limiting factor for the plant growth on the serpentine type of substrate. This was confirmed by later studies where the addition of Ca to the soil improved plant growth (Kruckeberg 1954; Vlamis 1949; Walker et al. 1955). Considering the above-mentioned, Donja Paklenica represents a real serpentine habitat, although more studies related to plant and microbial communities are necessary for a comprehensive perspective.

Bacterial isolates were collected solely from the *R. pseudoacacia* rhizosphere and roots. This plant spe-

Table VI
BLAST results after sequence editing of Rb and Eb sequences.

Isolate	Max score	Total score	Query cover	Query length	E-value	Percentage identity	Accession
Rb1-*Pseudomonas* sp.	1033	1033	100%	559	0.0	100.00%	MT555396.1
Rb2-*Pseudomonas* sp.	1029	1029	100%	567	0.0	99.47%	MT955582.1
Rb3-*Pseudomonas* sp.	1007	1007	98%	569	0.0	98.93%	MH018885.1
Rb4-*Pseudomonas* sp.	1026	1026	99%	560	0.0	99.64%	KJ598026.1
Rb5-*Pseudomonas* sp.	1035	1035	100%	560	0.0	100.00%	MT626826.1
Rb6-*Pseudomonas* sp.	1033	1033	99%	562	0.0	100.00%	PP738394.1
Rb7-*Pseudomonas* sp.	1024	1024	100%	558	0.0	98.82%	MN006008.1
Rb8-*Pseudomonas* sp.	1024	1024	100%	558	0.0	98.82%	MT373558.1
Rb9-*Bacillus* sp.	1037	1037	97%	573	0.0	100.00%	MG571235.1
Rb10-*Pseudomonas* sp.	1026	1026	99%	561	0.0	99.64%	HM755479.1
Rb11-*Pseudomonas* sp.	1026	1026	99%	560	0.0	99.64%	MH717340.1
Rb12-*Pseudomonas* sp.	1024	1024	100%	558	0.0	99.82%	MT280204.1
Rb13-*Pseudomonas* sp.	957	957	100%	518	0.0	100.00%	KT695830.1
Rb14-*Pseudomonas* sp.	1024	1024	100%	558	0.0	99.82%	MT533925.1
Eb1-*Bacillus* sp.	1040	1040	98%	574	0.0	100.00%	MT071453.1
Eb2-*Staphylococcus* sp.	1038	1038	100%	562	0.0	100.00%	KM021292.1
Eb3-*Bacillus* sp.	1033	1033	100%	559	0.0	100.00%	MT586023.1
Eb4-*Bacillus* sp.	992	992	100%	540	0.0	99.81%	MT032498.1
Eb5-*Bacillus* sp.	933	933	99%	506	0.0	100.00%	MW767008.1
Eb6-*Bacillus* sp.	963	963	100%	521	0.0	100.00%	MF375095.1
Eb7-*Pseudomonas* sp.	966	966	100%	523	0.0	100.00%	OM527246.1
Eb8-*Brevibacterium*/*Peribacillus*/*Bacillus* sp.	996	996	100%	555	0.0	98.93%	MT538263.1 KY323319.1
Eb9-*Lysinibacillus* sp.	942	942	100%	510	0.0	100.00%	OR104997.1
Eb10-*Bacillus* sp.	957	957	100%	518	0.0	100.00%	MF111532.1
Eb11-*Bacillus* sp.	1033	1033	100%	559	0.0	100.00%	MF111382.1

cies was selected based on its success in expansion and resilience to thrive in intolerable environmental conditions (Vlachodimos et al. 2013).

Heavy metal analysis generally showed Rb isolates tolerate Ni, Cu and Co. Serpentine environment likely exerts selective pressure on microbial communities, favoring those that can withstand metal toxicity (Sazykin et al. 2023). On the other hand, only a few endophytic isolates were resistant to the tested concentrations, which suggests endophytes may not be as well adapted to heavy metal stress as rhizobacteria. This could imply that while endophytes can benefit the host plant, they may be more sensitive to metal toxicity than rhizobacteria, which can thrive in metal-contaminated environments.

Siderophore production as a PGP trait in PGPB is well characterized in numerous studies (Rajkumar et al. 2010; Lee et al. 2012; Zhang et al. 2023). In our study, most high producers were from the *Pseudomonas* genus. *Pseudomonas* species as a plant growth-promoting bacteria can produce pyoverdine and induce plant growth (Gamalero and Glick 2011). In addition, they produce pyochelin, which can chelate various HMs such as Al, Cd, Co, Cr, Hg, Mn, Pb, Zn, and Ni (Braud et al. 2009). Although more research is required regarding physiological response, this may be one of the crucial components of *R. pseudoacacia* resilience in the serpentine environment. In some cases, the comparison between qualitative and quantitative analysis showed differences in siderophore production of the same isolate. This could be attributed to an inhibitory effect of HDTMA in CAS agar plates. Gram-positive bacteria are usually susceptible to HDTMA toxicity and can inhibit growth (Pérez-Miranda et al. 2007). Nye et al. (1994) also reported the toxicity of HDTMA to gram-negative soil bacteria in high concentrations. A Fe-deficient environment like an iron-free succinate medium employed in the quantitative method may stimulate siderophore production in some isolates (Kumar et al. 2017). Despite the occasional differences in results, Spearman's Rank Correlation Coefficient (R_s) revealed a positive correlation between the results of qualitative and quantitative analysis ($R_s = 0.64$; $p < 0.05$).

As one of the least available macronutrients in soil, phosphorus is a crucial element in plant metabolism. Considering the rapid transformation of P into insoluble forms unavailable to plants and the generally low concentration of P in serpentine soils, phosphate solubilizing bacteria (PSB) play a substantial role in phosphorus uptake by plants. Although no significant difference was observed between Rb and Eb, only four endophytic bacteria showed phosphate solubilizing properties. This case could be explained through the difference in contact availability of phosphorus in the rhizosphere and the inner tissue of the plant. In the screening of 82 isolates, Fan et al. (2018) discovered only three endophytic isolates with phosphate solubilization activity. To recruit PSB, roots exude diverse compounds necessary for the development of a productive microbial community in the rhizosphere (Berendsen et al. 2012; Wang et al. 2020). Previous studies showed less PSB presence than other PGPB isolates. Research conducted by Antoun et al. (1998) showed that out of 266 isolates, 54% had phosphate solubilization ability. At the same time, 83% of isolates showed siderophore production ability.

Given that *R. pseudoacacia* is a plant species from *Fabaceae*, it was obvious that many isolates exhibit nitrogen fixation (Masson-Boivin et al. 2009; Alemneh et al. 2020). Even though only the qualitative method was employed, we could monitor the difference between Rb and Eb. Firstly, the rapid change in color and formation of the pellicle was observed in Rb and then in Eb afterward. Eventually, almost all isolates from Rb and Eb expressed nitrogen fixation. It seems that Rb localization, composed mainly of *Pseudomonas* spp., and the production of nitrogenase simulates the intense response to nitrogen fixation through more accessible contact with N-forms (Haahtela et al. 1987; Wang et al. 2020).

All isolates produced IAA to a specific concentration, thus underlying the importance of this compound in *R. pseudoacacia* resilience. In our study, *Pseudomonas* and *Staphylococcus* isolates exhibit the highest concentration of IAA. Similarly, in the research of *Acacia farnesiana*, Herrera-Quiterio et al. (2020) found that these two genera, among 12%, identified with IAA production ability. In contrast, Yahaghi et al. (2018) investigated *Brassica juncea* and found that identified *Staphylococcus* isolates were not among IAA producers. Serpentine soil in our study was more similar to Herrera-Quiterio et al. (2020) while differed from Yahaghi et al (2018). It is also evident that the rate of IAA synthesis depends on locality and plant species (Barriuso et al. 2005).

The connection between IAA and ACC primarily involves their ability to regulate ethylene. The connection between IAA and ACC primarily involves their ability to regulate ethylene. As an ethylene precursor, ACC is involved in many processes, including abscission, fruit ripening, and response to various environmental stressors. The balance between IAA and ACC is crucial for plant overall health, and ACC deaminase plays a significant role in maintaining that balance in ethylene production. Almost all Rb isolates expressed ACC deaminase activity, while only five Eb showed this ability. Gamalero et al. (2023) highlight the common capacity of rhizospheric bacteria to produce ACC deaminase. Concurrently, in our study, many isolates produced both IAA and ACC deaminase. Fan et al. (2018) discovered the same trend, with 43 isolates from *R. pseudoacacia* roots exhibiting ACC deaminase activity and 50 producing IAA.

Similarly to our study where few *Pseudomonas* isolates revealed this trait, Shaharoona et al. (2006) also discovered this ability in *Pseudomonas*, where the improved growth of maize was observed following by *Pseudomonas* inoculation. This pattern, however, is not limited to *Pseudomonas* strains. In Eb isolates, this trait was detected in *Bacillus* and *Staphylococcus* sp. and other studies related to PGP properties (Araya et al. 2020; Misra and Chauhan 2020).

In the context of genus diversity, the difference was evident among rhizobacteria and endophytic bacteria. Endophytic bacteria represent a remarkable diversity reservoir and their significance in plant stress response has been mentioned in several studies (Brígido et al. 2019; Xu et al. 2019; Alibrandi et al. 2020). Rhizobacteria, on the other hand, demonstrated low diversity in our study, which may be explained by plant-soil feedback, which affects the microbial community in the soil. In addition, due to several challenging environmental factors, serpentine habitats are difficult for many plant species to thrive. Similarly, as a selective pressure, heavy metals and soil nutrients act as a driving force in the composition of rhizobacteria. Long-term exposure of soil bacteria to high concentrations of heavy metals leads to the emergence of heavy metal tolerance in the bacterial community. Soil bacteria are sensitive to heavy metal pollution; however, due to their great potential for adaptation, heavy metal-resistant strains manage to survive in these adverse conditions. As the abundance and the diversity of sensitive bacteria decrease while only resistant bacterial strains survive, heavy metals negatively affect the taxonomic structure and the diversity of the soil bacterial community (Sazykin et al. 2023). Sun et al. (2022) found relatively lower rhizosphere microbial diversity in severely contaminated soil, affecting microbial groups from Proteobacteria, Basidiomycota, Ascomycota, and Chloroflexi. In addition to high heavy metal concentrations, other properties of serpentine soils (texture, pH, low nutrient concentration, moisture, vegetation cover, etc.) may also negatively affect soil diversity. Root exudates strongly

impact the soil microbiota, acting as an attractant or repellent to particular bacterial and fungal strains (Hu et al. 2018). Plant-specific root and rhizosphere microbial communities may thus be defined by root exudates (Bulgarelli et al. 2013).

The serpentine bacteria exhibit remarkable plant growth-promoting (PGP) properties, such as nitrogen fixation, phosphate solubilization, and the production of growth-enhancing hormones like IAA, which may significantly contribute to *R. pseudoacacia* resilience in serpentine soils. These mechanisms improve nutrient uptake and promote plant vigor, particularly in the nutrient-poor and metal-rich conditions characteristic of serpentine environments. Although the effects of individual microbes on plant growth have been thoroughly studied, the impact of the serpentine microbiome has yet to be uncovered. In future studies, metagenomics could reveal specific microbial interactions and metabolic pathways that directly enhance *R. pseudoacacia* ability to cope with heavy metal stress and other environmental challenges.

R. pseudoacacia is an alien plant species with established invasiveness success. In addition to PGP serpentine bacteria, other factors could also support its spread. To have a broader perspective regarding its resilience, the following studies should encompass multiple serpentine localities and the physiological response of *R. pseudoacacia* to heavy metal stress in investigating potential phytoremediation applications.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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