



Characterization of rhizosphere and endophytic bacteria from roots of maize (*Zea mays* L.) plant irrigated with wastewater with biotechnological potential in agriculture

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ABSTRACT

The aim of this study was to characterize culturable rhizosphere and endophytic bacterial isolates isolated from rhizosphere soil and roots of maize plant irrigated with industrial and municipal wastewater in terms of resistance to heavy metals and salinity and plant growth promoting (PGP) traits. Results illustrated that both rhizosphere isolates and endophytic ones had various PGP characteristics in terms of both the number and the production amount of these characteristics. A substantial number of the bacterial isolates (both endophytic isolates and rhizosphere isolates) were tolerant to heavy metals (multi-metal resistant bacteria). Compared to endophytic isolates, rhizosphere isolates had greater resistance to heavy metals. Both endophytic isolates and rhizosphere ones showed remarkable resistance to salinity (7% NaCl). Based on comparison of 16S rRNA sequences and biochemical tests, the effective isolates, based on having multiple PGP characteristics and higher resistance to heavy metals and salinity, were identified. Isolates N5 and R7 were closely related to *Bacillus cereus* and *Enterobacter cloacae*, respectively. In addition, the ability of rhizosphere strain R7, as a multi-metal resistant bacterium, in the removal of cadmium (Cd) and lead (Pb) by its biomass and colonization of maize roots in the presence of these metals was evaluated. This strain could remove these metals from the solution (46.5–88.95%) and colonize both root surface and inside root of maize (4–7 Log₁₀ CFU (colony-forming unit) g⁻¹ fresh root weight) under heavy metal stress. Therefore, it can be concluded that maize plant irrigated with industrial and municipal wastewater harbors salinity and heavy metals-resistant bacteria and may be potential reservoirs for isolating bacteria effective at alleviating heavy metal stress in the plant, reducing accumulation of heavy metals in crops such as maize, and removing heavy metals in aqueous media (bioremediation of heavy metal-contaminated wastewater system).

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1. Introduction

A diverse range of bacteria live in the rhizosphere and various organs (e.g., roots, stems, leaves, seeds, and fruits) of plants [1]. Beneficial bacteria associated with the plants play an important role in the health and growth of plants under various environmental stresses [2]. Bacteria colonizing the roots of plants and have beneficial effects on plant growth and development are known as plant growth promoting–rhizobacteria (PGPR) [3]. These bacteria benefit plant growth by various mechanisms [2]. Endophytic bacteria, which colonize the internal tissue (the intercellular spaces) of plants without indicating any negative influence on their

host, constitute a great reservoir of bacterial diversity with a striking biotechnological potential [1]. According to a previous study [4], bacterial community inhabiting rhizosphere are also a source of formation of the community of endophytic bacteria. It is known that the mechanisms used by endophytic bacteria to improve plant growth are similar to those of rhizospheric bacteria. Further, compared to rhizobacteria, the endophytic bacteria can also diminish the adverse effects of environmental stresses on plants effectively [2].

New advance in plant–bacteria interactions research reveals that plants are able to shape their rhizosphere and endorhiza microbiome [5] and recruit environmental stress-resistant bacteria that contain specific characterization. Under stress conditions, plants need bacteria for their growth and establishment in different ecosystems [6]. Symbiotic bacteria exist in all plants living in the natural ecosystems. This relationship may be a key factor involved in plant's stress-tolerance ability. Indeed, the

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genetic differentiation that exists in bacteria associated with plants help local adaptation of the plants to their environment [7]. It has been proven that transplanting different plant species in the absence of bacteria is notoriously difficult [8], which hints at a role of bacteria in plant growth under stressful conditions.

At the present time, the problem of water scarceness is one of the greatest problems for humanity throughout the world. In this situation, the treatment and re-rotation of wastewater may be as one of the most important solution in the development of water resources management, which can also play a momentous role in relation to water scarcity problems in agriculture. However, irrigation with wastewater has significantly augmented the accumulation of heavy metals in soil, groundwater and plants [9,10]. Heavy metals are not easily eliminated, and cannot be chemically or biologically degraded, making their toxicity long-lasting. Some heavy metals are essential to different metabolic activities and are needed by organisms at low concentrations [11,12], but above a certain threshold, these metals in soils negatively impress the composition of plant and microbial communities [12–14]. Toxic heavy metals cause soil salinity, nutrient deficiencies and imbalance by interfering with essential nutrient uptake and distribution in plants. These metals also transfer into the food chain through plants grown on soils contaminated with the heavy metals [12].

There are reports suggesting that rhizosphere and endophytic bacteria, in addition to alleviating the harmful effects of heavy metal toxicity stresses in plants [2], can, through various mechanisms such as biosorption/bioaccumulation, reduce the availability, the absorption and accumulation of heavy metals in plants [12]. The use of heavy metals-resistant beneficial bacteria associated with plant roots (rhizosphere and endophytic bacteria) in harsh environments could be a practicable strategy to deal with limitations caused by heavy metal pollution to agricultural production [12]. It is known that the ability and efficiency of beneficial bacteria in transforming nutrients and increasing plant tolerance to environmental stresses are associated with the ecological conditions including the climate, weather conditions, soil characteristics (e.g., high salinity, presence of heavy metals, etc.), and interaction with other indigenous microbial flora in the soil [15]. For example, the performance of phosphorus-solubilizing microorganisms (PSM) is severely affected by environmental factors especially stress factors [16,17] including heavy metal stress. Detailed knowledge concerning characteristics of the bacterial community associated with maize (*Zea mays* L.) plant, as a direct staple food for millions of individuals but with a high irrigation requirement, irrigated with industrial and municipal wastewater is limited. To effectively utilize the rhizosphere and endophytic bacteria (e.g., for use in alleviating heavy metal stress and salinity stress caused by application of wastewater for irrigation and in reducing the heavy metal accumulation in maize plant), it is important to characterize culturable rhizosphere and endophytic bacteria isolated from this plant in terms of resistance to heavy metals and salinity and plant growth promoting (PGP) traits.

Therefore, this study addressed a fundamental question: How do the traits (e.g., resistance to heavy metals and salinity and PGP traits) of bacterial isolates differ between the rhizosphere and endorhiza of maize irrigated with industrial and municipal wastewater? In order to address this question, all culturable rhizosphere and endophytic bacterial isolates isolated from the maize plant were screened for their resistance to heavy metals and salinity and PGP traits. In addition, the ability of an effective isolate (a multi metal-resistant bacterium) in removal of Cd and Pb and colonization of maize plant roots in the presence of these metals was also evaluated. The outcome obtained from this study provided insights into the relationships between the rhizosphere

and endophytic bacteria in the rhizosphere and endorhiza of maize plant irrigated with industrial and municipal wastewater.

2. Materials and methods

2.1. Isolation of endophytic and rhizosphere bacteria

A total of 20 samples of rhizosphere soil and roots from maize (c.v., KSC-703) plants grown at maize fields (53° 96' 47" North, 93° 33' 19" East, and 937 m above sea level, located in Golhesar area in Shahrerey, Tehran, Iran), irrigated with industrial and municipal wastewater and irrigated with normal water (as control treatment) were randomly collected. The presence of heavy metals such as lead (Pb), 2.25 mg L⁻¹; manganese (Mn), 0.06 mg L⁻¹; iron (Fe), 0.6 mg L⁻¹; cadmium (Cd), 0.018 mg L⁻¹; zinc (Zn), 0.77 mg L⁻¹; chromium (Cr), 0.01 mg L⁻¹; and nickel (Ni), 0.1 mg L⁻¹ in the wastewater has been reported. The soil in which the maize plant was planted had been irrigated with the wastewater for ten years. Continuous irrigation of the agricultural land by wastewater during these years increased heavy metals content in maize plants (higher than the permissible limit). Soil samples were analyzed to characterize physical, chemical and biological traits. Some of physical, chemical and biological traits of field soil included: The soil texture (27% clay, 33% silt, and 40% sand), loam; pH, 7.63; electrical conductivity (EC) of saturated paste extract, 3.43 dS m⁻¹; organic carbon, 5.1 g kg⁻¹; total nitrogen (N_{tot}), 4.4 g kg⁻¹; calcium carbonate equivalent (CCE), 110 g kg⁻¹; available potassium (K_{avail}), 245 mg kg⁻¹; sodium, 13.5 mg kg⁻¹, available phosphorus (P_{avail}), 10.2 mg kg⁻¹; and culturable bacterial population, 4.7 × 10⁶ CFU (colony-forming units) g⁻¹. These traits were measured according to a previous method [18]. The healthy plants at flowering stage were randomly collected from different positions in the maize fields (2 ha) and transferred to laboratory in special iceboxes for isolating and purifying bacterial isolates. Root endophytic and rhizosphere bacteria were isolated according to the method suggested by Etesami et al. [19]. After preparing the dilution series from the samples, 0.1 mL of each dilution of 10⁻⁴ to 10⁻⁷ was spread on plates containing nutrient agar (NA) culture medium. All plates inverted were placed for 3–5 days at 28 ± 2 °C in incubator and the number of colonies appearing on the plates was counted. The number of isolated endophytic and rhizospheric bacteria was reported as CFU g⁻¹ fresh root weight and soil weight, respectively. The purification steps of these isolates were carried out on the same medium through sub-culturing. Based on phenotypic features (i.e., color, shape, motility, growth rate, and colony morphology) and Gram-staining, the bacterial isolates were grouped and stored in a refrigerator at 4 °C for subsequent study. In addition, to maintain the isolates for long-term, they were stored in nutrient broth (NB) containing 20% glycerol at –80 °C. In order to hinder the growth of fungi, cycloheximide (100 mg L⁻¹) was added to culture media.

2.2. Preparation of bacterial inocula

To perform each of the tests in this study, the fresh inoculum of each isolate was prepared in the following way. Initially, rhizospheric and endophytic bacterial isolates were re-cultured on NA medium. The 100 mL-Erlenmeyer flask containing 20 mL of sterilized NB culture medium were inoculated with bacterial isolates. The cultures were incubated at 28 ± 2 °C for 48 h (for fast-growing bacteria) and 72 h (for slow-growing bacteria) on a shaker with a rotation speed of 120 rpm until (turbid) logarithmic phase was attained 5 × 10⁸ cells mL⁻¹. The bacterial cells were harvested through centrifugation (7000 g for 10 min) after growth in NB culture medium and then washed twice with phosphate buffer and then suspended in the same buffer.

2.3. Assay of bacterial isolates in terms of resistance to heavy metals

In this assay, resistance of all rhizosphere and endophytic isolates to heavy metals Cd, Pb, Cr, cobalt (Co), Ni, copper (Cu), and Zn with varying concentrations of 0.5, 1, 1.5, 2, 2.5, 3, and 3.5 mM was studied. This assessment and the concentrations of the selected heavy metals were carried out according to a previous report [20]. For this purpose, each isolate was cultured on minimal salt medium (MSM), which is composed of 3 g K_2HPO_4 , 6 g Na_2HPO_4 , 6 g NaCl, 2 g NH_4Cl , 0.1 g $MgSO_4$, 8 g glucose per liter at pH 7.2, containing different concentrations of heavy metal in a streak manner. In this culture medium, Cd as $CdCl_2$, Pb as $PbCl_2$, Cr as $Cr(NO_3)_3$, Co as $CoCl_2 \cdot 6H_2O$, Ni as $NiCl_2 \cdot 6H_2O$, Cu as $CuCl_2$, and Zn as $ZnCl_2 \cdot H_2O$ were used. All metal salts (purity 99%) used in this study were obtained from Merck. The cultured plates were incubated at $28 \pm 2^\circ C$ for 24 to 72 h (depending on their growth rate) after sealing. The colonies that could tolerate concentrations of heavy metals were selected and their growth was compared to the growth of which in control plates. For each isolate, three replicates were taken. Additionally, minimum inhibitory concentration (MIC) of the heavy metals to these isolates was also determined.

2.4. Salt tolerance test of isolates

For this purpose, NA medium containing different salinity percentages (1–10% using NaCl salt) was used. At first, culture media including different concentrations of NaCl were distributed in sterile conditions in Petri dishes and fresh culture of each isolate with a uniform population (5×10^8 cells mL^{-1}) was spotted onto the agar plates. For each isolate, three replicates were taken and the petri dishes were kept in the incubator at $28 \pm 2^\circ C$ after sealing. Changes in diameter, status and appearance of colonies were investigated after 3, 6, and 9 days after incubation. The tolerance of isolates to different levels of salinity was assessed by observing the quality of the colonies grown in the control plates [21].

2.5. Screening isolates for PGP traits

The ability of production of indole-3-acetic acid (IAA) by these isolates was evaluated according to the method described by Patten and Glick [22], based on a colorimetric technique with Van Urk Salkowski reagent using the Salkowski's method. IAA production ($\mu g mL^{-1}$) was observed as the development of a pink-red color, and the absorbance was measured at 530 nm using Spectrophotometer (Shimadzu-UV3100). Siderophore production by the isolates was determined qualitatively using chrome azurol S (CAS) agar as described by Schwyn and Neilands [23]. Yellow-orange halos formed around the colonies on blue agar were considered indicative of siderophore production. Qualitative assay of insoluble inorganic phosphate solubilization by bacterial isolates was determined by the method described by Sperber [24] on agar plates including Sperber's medium supplemented with 2.5 g calcium phosphate as the only source of phosphorus. Siderophore production and insoluble phosphate-solubilization index were evaluated according to the ratio of the halo diameter (HD, in mm) to the colony diameter (CD, in mm) of bacterial isolates on the relevant culture medium. Qualitative assay of ACC deaminase production by bacterial isolates was determined by the method described by Penrose and Glick [25] on Dworkin and Foster (DF)-salts minimal medium supplemented with 3 mmol L^{-1} ACC as the only nitrogen source. Growth of isolates, colony diameter (CD, in mm), on ACC-supplemented DF-plates was considered as an index for evaluating ACC deaminase-producing isolates. The experiments were done in triplicates.

2.6. Molecular identification of the selected effective isolates

In this stage, among rhizosphere and endophytic isolates, an endophytic isolate (N5) as the representative of endophytic isolates and a rhizosphere isolate (R7) as the representative of rhizosphere isolates were selected. This selection was based on resistance of bacterial isolates to heavy metals and salinity and having multiple PGP characteristics. The selected isolates showed high degree of resistance to heavy metals and salinity and produced all four PGP traits. Based on morphological characteristics, biochemical tests, and sequencing of 16S rRNA gene, the isolate N5 and isolate R7 were identified. Genomic DNA of these isolates was extracted from these isolates using isolation kit (Promega, Madison, WI, USA). The amplification of 16S rRNA gene was done using universal primers 27F and 1492R following the protocol described by Edwards et al. [26]. The PCR products were sequenced at Macrogen Inc., Republic of Korea, and the obtained sequences were compared to those from the GenBank using the BLAST program. The phylogenetic tree was constructed using the Neighbor Joining method using the Mega 5 software. The nucleotide sequences identified in this study were sent to GenBank databases and recorded with various accession numbers.

2.7. Assay of growth rate of isolates N5 and R7 in the presence of Cd and Pb

In this assay, heavy metals Pb and Cd were selected as the representative of heavy metals because these two metals are frequently found in most industrial and municipal wastewaters. Viable plate count method was used to evaluate and determine the growth curve of selected bacterial isolates in the presence of 2 mM Cd, 2 mM Pb, and 2 mM Cd + 2 mM Pb at different times (0–120 h). To carry out this assessment, each isolate was grown in 100 mL-Erlenmeyer flasks including NB medium supplemented with 2 mM Cd, 2 mM Pb, and 2 mM Cd + 2 mM Pb separately at $28 \pm 2^\circ C$ on a rotary shaker at 120 rpm for 120 h. Each isolate was tested using a minimum of three replications per treatment. In this assay, the isolates grown in 100 mL-Erlenmeyer flasks including NB medium non-supplemented with these heavy metals were considered as control. Finally, after making dilution series, 0.1 mL of suspension of each bacterial isolate grown in the presence of the heavy metals was spread on NA plates containing concentrations of the heavy metals (2 mM Cd and 2 mM Pb). After incubating the plates at $28 \pm 2^\circ C$ for 24 h, the bacterial growth curves were plotted by counting the colonies grown on the plates containing heavy elements (2 mM Cd and 2 mM Pb) at 24-h intervals.

2.8. Evaluation of removal of Cd and Pb by isolate R7 biomass

Since endophytic isolate N5 was not able to grow in the presence of 2 mM Cd in the liquid medium, only rhizosphere isolate R7 was selected for this evaluation. In order to carry out this assessment, the isolate R7 (a volume of 1.0% of a cell suspension grown for 16 h from NB medium) was inoculated to NB-medium containing concentrations (2 mM) of Cd and Pb separately and in combination as described above. Cell-free sets were retained to assess the artifacts might ascend due to the metal sorption on the glass surface of the container. After 48 h of incubating at $28 \pm 2^\circ C$ on a shaker at 120 rpm and harvesting the cells using centrifugation (7000 g for 10 min), the percentage of the metal removal (R) by the biomass of this isolate was calculated using Eq. (1):

$$R (\%) = (C_i - C) / C_i \times 100 \quad (1)$$

Where, C_i and C are the initial concentration of the metal in $mg L^{-1}$ and the concentration of the metal in the supernatant in $mg L^{-1}$

after 48 h, respectively. The concentration of metals was read by atomic absorption spectrometry (Shimadzu-AA6400, Japan). The experiment was done in triplicates.

2.9. Maize colonization assay by isolate R7 in the presence of Cd and Pb

This evaluation was carried out according to the method described by Etesami and Alikhani [27] with some modification. Briefly, the disinfected maize seeds were germinated in Petri dishes containing 1% water-agar in an incubator at $28 \pm 2^\circ\text{C}$. When the length of all the seedlings reached 0.5 cm, the seedlings were transferred into the Falcon tubes cut off from the bottom and suspended in sterilized glass jars containing 60 mL of Hoagland solution supplemented with 5 mg L^{-1} Cd and 100 mg L^{-1} Pb. The sterility conditions were met throughout this assessment. The sterilized-glass jars containing the seedlings were incubated in the dark at $28 \pm 2^\circ\text{C}$ for 3 days for more germination. The $100\ \mu\text{L}$ of suspension of the isolate R7 (5×10^8 cells mL^{-1}) was inoculated on seedlings. Control plants were inoculated with $100\ \mu\text{L}$ of sterilized-phosphate buffer. Then glass jars were maintained in a growth chamber with a brightness of 10 to 12000 lx (14 h in light and 10 h in darkness) at $28 \pm 2^\circ\text{C}$ for 40 days. At the end of this period, the population size of this isolate on root surface (rhizoplane) and inside root (endophyte) was measured and recorded. Dilution series made from the resulting solution were spread on NA plates containing 2 mM Pb and 2 mM Cd, and after 2 to 3 days of growth at $28 \pm 2^\circ\text{C}$, the number of bacterial isolates were counted and considered as rhizoplane and endophytic bacterial isolates. The experiment was done in triplicates.

2.10. Statistical analysis

In a completely randomized design (CRD), all of the tests were arranged with three replications in each treatment. Normality test of data was done. Using SAS computer programs (SAS Institute, Cary, NC, USA); all data were subjected to analysis of variance (ANOVA). The data were presented as means \pm SE (standard error).

3. Results

3.1. Isolation of rhizosphere and endophytic bacteria

Rhizosphere and endophytic bacterial isolates isolated from the maize plants non-irrigated with industrial and municipal wastewater (control plants) were mainly susceptible to heavy metals, which demonstrate that the quality of wastewater has had an impact on the endophytic or rhizospheric microbial community (data not shown). A total of 170 rhizospheric and endophytic bacterial isolates were isolated from roots of maize plant irrigated with industrial and municipal wastewater. Based on the isolation source of these isolates, from these 170 isolates, 110 were rhizosphere isolates (65%) and 60 were root endophytic isolates (35%). The population size (CFU) \pm SE of the isolates in the rhizosphere and endorizha of maize plant was $3.4 \pm 2.12 \times 10^6$ and $6.8 \pm 1.20 \times 10^3$, respectively. Gram-staining and KOH test on these isolates showed that 52% of these isolates were Gram-positive and the remaining isolates were Gram-negative.

3.2. Heavy metals-resistant rhizosphere and endophytic bacteria

The abundance of heavy metals-resistant endophytic and rhizosphere isolates is shown in Fig. 1A–G. Based on the results of this assay, with increasing the metal concentrations (except for Cr) in the culture medium, the percentage of tolerant rhizosphere and endophytic bacteria decreased. The heavy metal tolerance test indicated that the MIC of rhizosphere isolates reached 3 mM Pb, 2.5 mM Cd, 3.5 mM Cu, 0.5 mM Co, 3 mM Ni, higher than 3.5 mM Cr, and 3 mM Zn,

respectively. In addition, this test showed that the MIC of endophytic isolates reached 3.5 mM Pb, 0.5 mM Cd, 1 mM Cu, 0.5 mM Co, 2.5 mM Ni, higher than 3.5 mM Cr, and 3 mM Zn, respectively. Cd was highly toxic for the endophytic strains and was lethal even at low concentrations (Fig. 1B). Results also showed that 100% of rhizosphere and endophytic bacteria tolerate 0.5 to 3.5 mM Cr (Fig. 1F) but none of them were able to grow with 0.5 to 3.5 mM Co (Fig. 1D). It should be mentioned that endophytic and rhizosphere isolates were particularly tolerant to several elements at the same time. However, bacterial isolates that showed the capacity to tolerate high heavy metal concentrations were isolated from the rhizosphere of maize plant. In general, the resistance of endophytic bacterial isolates to heavy metals evaluated in this study was reduced as follows:

$\text{Cr} > \text{Pb} > \text{Zn} > \text{Ni} > \text{Cu} > \text{Cd} = \text{Co}$

And the resistance of rhizosphere bacterial isolates to heavy metals evaluated in this study was reduced as follows:

$\text{Cr} > \text{Cu} > \text{Pb} = \text{Zn} > \text{Ni} > \text{Cd} > \text{Co}$

3.3. Salinity-resistant rhizosphere and endophytic bacteria

The abundance of salinity resistant endophytic and rhizospheric isolates is shown in Fig. 2. The results of this assessment showed that a significant percentage of endophytic and rhizospheric isolates was resistant to low salinity percentages (1 and 3%). The 25 and 45% of rhizospheric and endophytic isolates were able to grow in culture medium containing 5 and 7% NaCl, respectively. In addition, none of the rhizosphere and endophytic isolates was able to grow in 10% NaCl medium. In general, the growth of isolates decreased with increasing NaCl concentration in all endophytic and rhizosphere isolates.

3.4. PGP traits of rhizosphere and endophytic bacteria

The total potential of 170 isolates was evaluated for PGP characteristics. The abundance of the PGP characteristics of rhizospheric isolates and endophytic isolates is shown in Fig. 3A and B, respectively. The 72 isolates out 110 rhizosphere isolates produced IAA, while 45 isolates from 60 endophytic isolates were positive for IAA production. The percentage of IAA-producing endophytic isolates (45/60) was higher than the percentage of IAA-producing rhizosphere bacteria (72/110). About 46.9% of the endophytic isolates produced siderophore, 56.5% produced ACC deaminase, and 35.4% of these isolates were able to solubilize mineral phosphate, while only 53.6% of the rhizosphere isolates produced siderophore, 56.3% showed the activity of ACC deaminase, and 41.8% of these isolates were capable of solubilizing inorganic phosphate. Of the 110 rhizosphere isolates, 59 isolates showed an orange halo surrounding colonies on the CAS medium. The 46 isolates from 110 isolates were able to solubilize phosphate. On the other hand, sixty-two isolates were able to use ACC as the sole source of nitrogen due to the ACC deaminase production.

The evaluated isolates also produced different amounts of siderophore, IAA, and ACC deaminase. They also showed different ability to solubilize phosphate. The abundance of production amount of siderophore (Fig. 4A), phosphate solubilization (Fig. 4B), IAA (Fig. 4C), and ACC deaminase (Fig. 4D) by endophytic and rhizospheric isolates, which were positive in terms of these PGP traits, is shown in Fig. 4. These results showed that these isolates produced IAA in the range of 4.23 ± 0.33 – $47.30 \pm 0.48\ \mu\text{g mL}^{-1}$ in the presence of $100\ \mu\text{g mL}^{-1}$ of L-Tryptophan (L-Trp) (Fig. 4C). These isolates also showed differences in PGP traits. In addition to the high prevalence of IAA-producing isolates among endophytic isolates, a higher percentage of these isolates with high ($>20\ \mu\text{g mL}^{-1}$) and medium (10 – $20\ \mu\text{g mL}^{-1}$) production ability was found in the

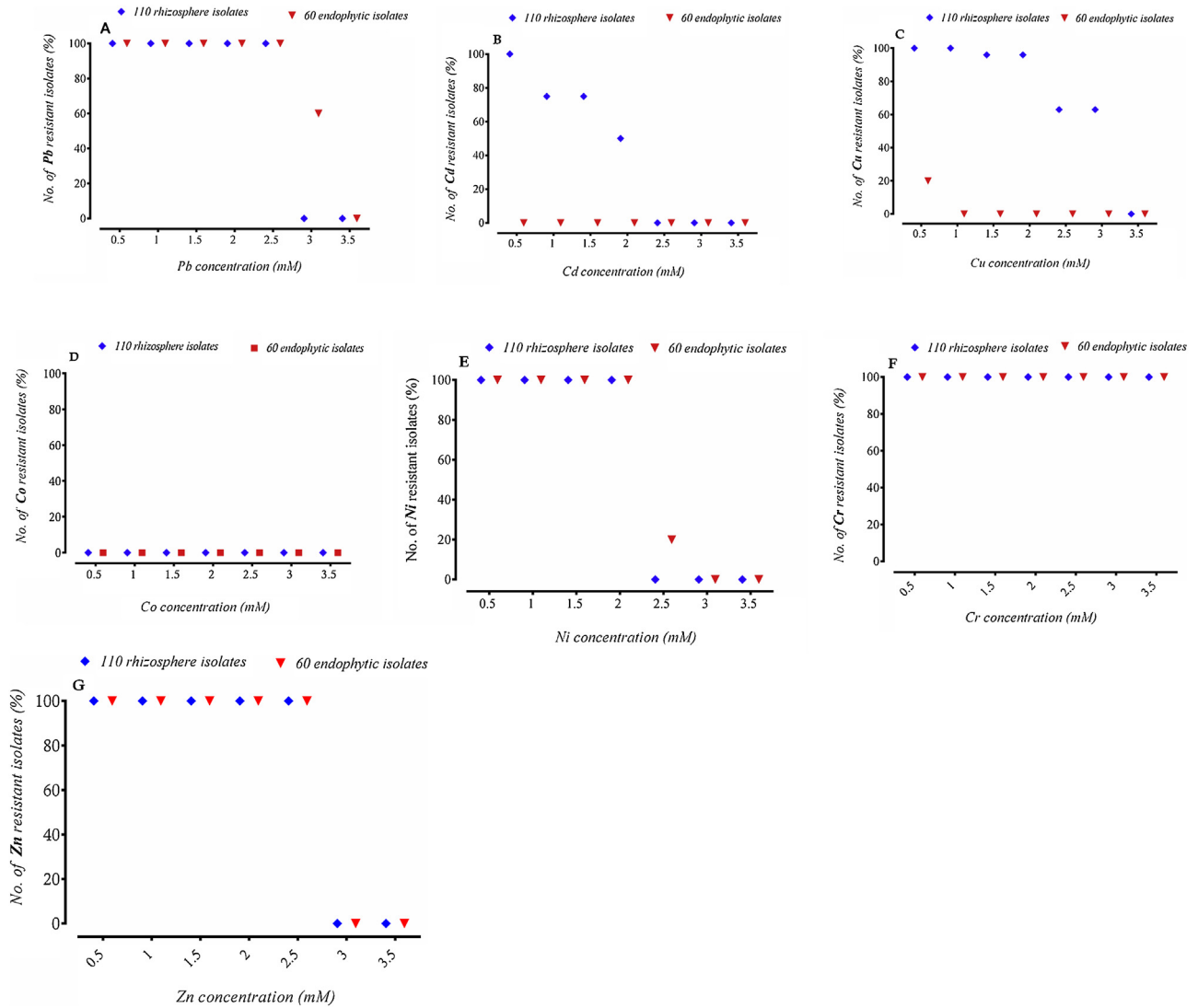


Fig. 1. Abundance of endophytic and rhizosphere bacterial isolates resistant to different concentrations of heavy metals isolated from maize (*Zea mays* L.) plant irrigated with industrial and municipal wastewater.

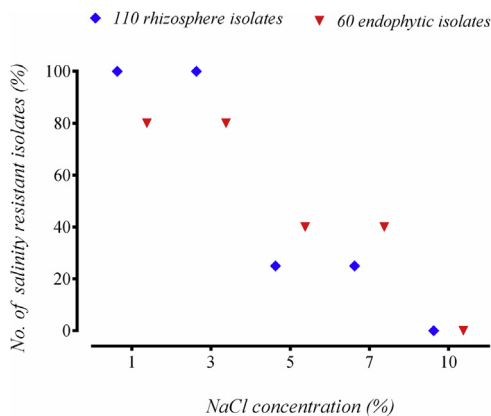


Fig. 2. Abundance of endophytic and rhizosphere bacterial isolates resistant to salinity isolated from maize (*Zea mays* L.) plant irrigated with industrial and municipal wastewater.

isolation source (Fig. 4C). These results showed that an important part of all isolates showed the ability to produce IAA. In addition, the percentage of phosphate-solubilizing endophytic (35.4%) and rhizosphere isolates (41.8%) was not similar. The abundance of siderophore-producing rhizospheric isolates was more than the number of siderophore-producing endophytic isolates. Generally, the percentage of IAA, ACC deaminase, siderophore and phosphate solubilization-containing bacteria in both isolation sources was reduced as follows: production of IAA > production of ACC deaminase > production of siderophore > phosphate solubilization.

3.5. Identification of the effective endophytic and rhizosphere isolates

The characteristics of these isolates are presented in Table 1. Databases in the GeneBank for the sequences similar to the 16S rRNA sequences of these isolates were investigated. Identification of the 16S rRNA gene of the endophyte isolate N5 and rhizosphere isolate R7 showed that these isolates are closely related to *Bacillus cereus* and *Enterobacter cloacae*, respectively. The 16S rRNA sequences of endophytic and rhizospheric isolates were similar to *B. cereus* M2 (accession number KP895573) and *E. cloacae*

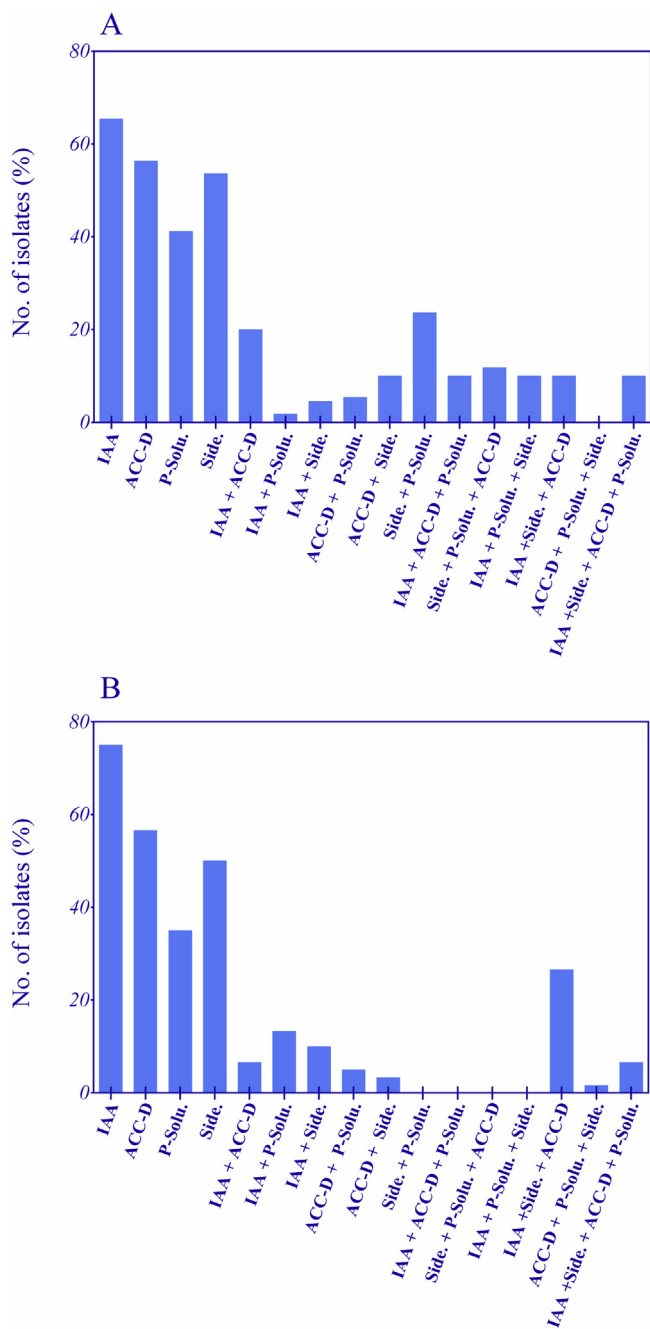


Fig. 3. Abundance of PGP activities of rhizosphere bacterial isolates (A) and endophytic bacterial isolates (B) isolated from maize (*Zea mays* L.) plant irrigated with industrial and municipal wastewater.

SKUAST3 (accession number KY612285) on the BLAST search in NCBI. The nucleotide sequences identified in this study were sent to the Gene Bank database and recorded with accession number MF687205 for strain R7 and accession number MF687206 for strain N5. The phylogenetic tree of the identified bacterial isolates in the research is shown in Fig. 5. It was apparent from the phylogenetic tree that isolates N5 and R7 exhibited a close relation with their nearest neighbours (*B. cereus* and *E. cloacae*).

3.6. The growth rate of effective strains in the presence of Cd and Pb

The growth pattern of *B. cereus* N5 and *E. cloacae* R7 grown in a NB medium containing 2 mM Cd and 2 mM Pb separately and in

combination is shown in Fig. 6. As seen in Fig. 6A, the N5 strain was able to grow well in the presence of 2 mM Pb and had a similar growth trend to control. *E. cloacae* R7 could well grow in the liquid medium in the presence of Pb, Cd, and Pb and Cd simultaneously (Fig. 6B). In this assessment, like evaluating the strain in the NA medium containing heavy metals, Cd was more toxic to the strain than Pb.

3.7. Assessment of removal of Cd and Pb by biomass of strain R7

The percentage of removal of Cd and Pb by strain R7 biomass is shown in Fig. 6C. As shown in Fig. 6C, this strain was able to remove a higher percentage of Pb than Cd from the liquid medium. In addition, when Pb was used with Cd in combination, the strain could remove 88.95% of Pb from the liquid medium. In general, the removal of metals from the solution was reduced as follows: Cd in the absence of Pb (46.5%) < Cd in the presence of Pb (58%) < Pb in the absence of Cd (75.04%) < Pb in the presence of Cd (88.95%).

3.8. Maize plant colonization by *E. cloacae* R7 in the presence of Cd and Pb

In order to determine whether the rhizosphere strain R7 had the ability to colonize and maintain a high level of population on maize roots, a study was conducted with a controlled system in the presence of 5 mg L⁻¹ Cd and 100 mg L⁻¹ Pb. The results of this assessment showed that this strain had a high ability to colonize root surface under both heavy metal stress conditions and non-stress conditions. In this study, in non-inoculated control plants, no bacterium from rhizoplane and inside roots was isolated. In addition, this strain was able to colonize inside tissues of root. The endophytic and rhizoplane population size of this strain in the absence of Cd and Pb (controls) was about 3 and 6 Log₁₀ CFU g⁻¹ fresh root weight, respectively, while the endophytic and rhizoplane population size of this strain in the presence of Cd and Pb was about 4 and 7 Log₁₀ CFU g⁻¹ fresh root weight. In maize plants inoculated with strain R7 under heavy metal stress, there was a significant increase in root hairs and root length compared to non-inoculated control plants and the inoculated non-stressed plants (data not shown). These results also showed that no signs of disease and wilting were observed in maize seedlings inoculated with this strain.

4. Discussion

New advance in plant–bacteria interactions research revealed that plants are able to shape their rhizosphere and endorhiza microbiome [5] and recruit environmental stress-resistant bacteria that contain specific characterization. Under stress conditions, plants need bacteria for their growth and establishment in different ecosystems [6]. The results of this study showed that rhizosphere and endorhiza of maize plants irrigated with industrial and municipal wastewater harbor heavy metals-resistant bacteria with multiple PGP traits. In previous studies, the presence of rhizospheric bacteria or rhizoplane bacteria (on the root surface) as well as endophytes in plants grown in soils contaminated with heavy metals has also been reported [28,29]. For example, in a study [30], 96 endophytic isolates were isolated from *Solanum nigrum* L. grown in soils contaminated with heavy metals. It has been well established that no plant can grow well without the presence of micro-organisms [31]. In this study, endophytic and rhizosphere bacteria associated with maize plant were likely to contribute to the growth and tolerance of this plant to environmental stresses because there were no symptoms of toxicity of heavy metals and wilting on the sampled maize plants and these plants were completely healthy. These results also showed that the number of isolates obtained from

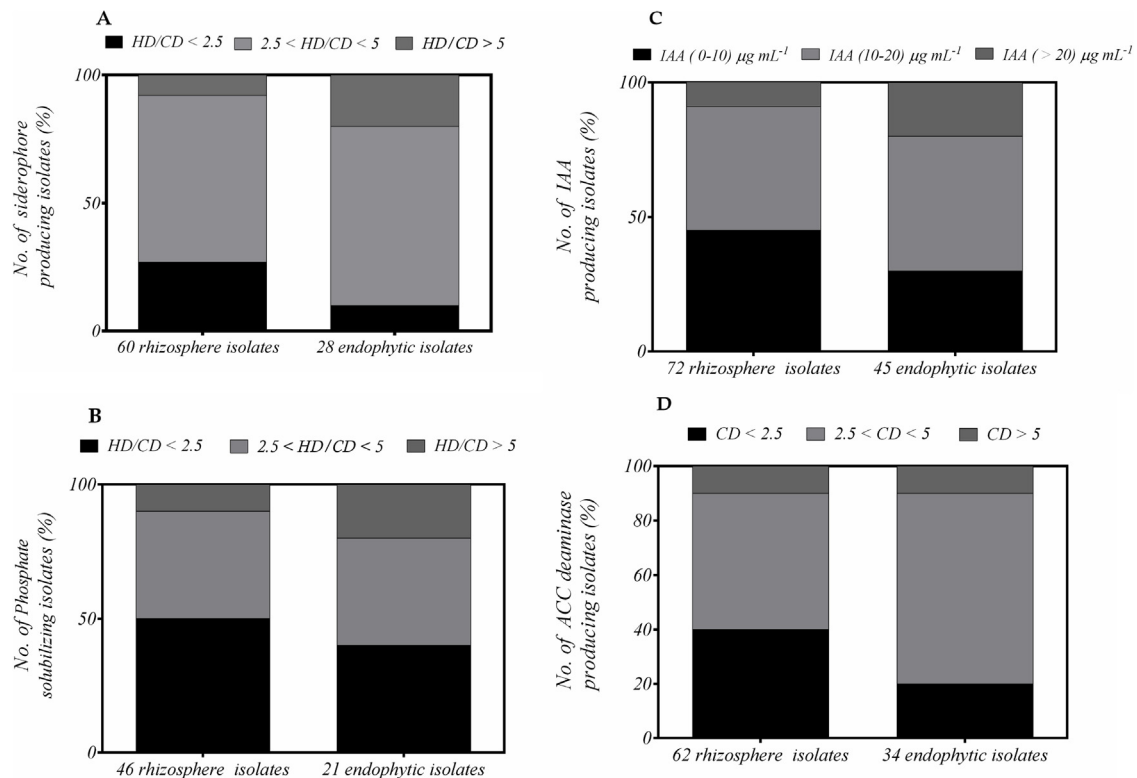


Fig. 4. Abundance of the production amount of four PGP traits by endophytic and rhizosphere bacterial isolates isolated from maize (*Zea mays* L.) plant irrigated with industrial and municipal wastewater. Legends show; CD: Colony diameter (mm) of isolates grown on Dworkin and Foster (DF)–salts minimal medium containing ACC, HD/CD: halo diameter to colony diameter of isolates. Siderophore production: low production (HD/CD < 2.5), medium production (2.5 < HD/CD < 5), and high production (HD/CD > 5) (A); phosphate assay: low production (HD/CD < 2.5), medium production (2.5 < HD/CD < 5), and high production (HD/CD > 5) (B); IAA assay: low production (0–10 $\mu\text{g mL}^{-1}$), medium production (10–20 $\mu\text{g mL}^{-1}$), and high production (>20 $\mu\text{g mL}^{-1}$) (C); and ACC deaminase production: low production (CD < 2.5), medium production (2.5 < CD < 5), and high production (CD > 5) (D).

the rhizosphere was higher than that of the isolates of root. The population size of the culturable endophytic bacteria in the roots was lower than that of the rhizosphere bacteria. Generally, endophytic bacteria are found in lower population sizes than rhizospheric bacteria and bacterial pathogens [32,33]. In addition, there were an equal number of Gram–negative and Gram–positive bacteria among these isolates. These results are consistent with the findings of other researchers [34,35].

In this study, almost all rhizosphere and endophytic bacteria were resistant to most heavy metals with different concentrations. Past studies have shown that microorganisms have already been adapted to severe conditions such as heavy metal toxicity and other requirements for their survival in places contaminated with metals [36]. Rajkumar et al. [37] also stated that bacterial isolates isolated from contaminated environments have higher resistance to heavy metals than isolates isolated from non–contaminated

Table 1

Morphological, biochemical and PGP traits and MIC of the endophytic strain N5 and rhizosphere strain R7 isolated from endorhiza and rhizosphere of maize (*Zea mays* L.) plant irrigated with industrial and municipal wastewater.

Isolate	Accession number	Morphological and biochemical traits	Closest relative (accession number)	Similarity (%)	PGP traits	MIC values of the test metals (mM)
N5	MF687206	Motile, rod, G+, spore formers (+), fast grow, catalase (+), oxidase (+), nitrate reduction (+), arginine dehydrogenase (+), urease test (+), amylase production (+), gelatinase production (+), casein hydrolysis (+), citrate (+), KOH test (–), lecithinase test (+), methyl red (–), phosphatase (+), glucose fermentation (+), mannitol fermentation(–), sucrose fermentation(+), arabinose fermentation (–), ammonia production (+), growth in NaCl 7% (+), and growth at 50 °C (+)	<i>Bacillus cereus</i> M2 (KP895573)	99	IAA (47.3 $\mu\text{g mL}^{-1}$), ACC deaminase (+), siderophore (+), and phosphate solubilization (+)	Cd (0.5), Pb (3.5), Ni (2.5), Co (0.5), Cu (1), Zn (3), and Cr (>3.5)
R7	MF687205	Motile, small rod, G–, spore formers (–), fast grow, catalase (+), oxidase (–), nitrate reduction (–), arginine dehydrogenase (+), urease test (–), amylase production (–), gelatinase production (+), casein hydrolysis (+), citrate (+), KOH test (+), lecithinase test (+), methyl red (–), phosphatase (+), glucose fermentation (+), mannitol fermentation(+), sucrose fermentation(+), arabinose fermentation (+), ammonia production (+), growth in NaCl 7% (+), and growth at 50 °C (–)	<i>Enterobacter cloacae</i> SKUAST3 (KY612285)	99	IAA (35.4 $\mu\text{g mL}^{-1}$), ACC deaminase (+), siderophore (+), and phosphate solubilization (+)	Cd (2.5), Pb (3), Ni (2.5), Co (0.5), Cu (2.5), Zn (3), and Cr (>3.5)

The presence of an activity is indicated by “+”, and the absence is indicated by “–”; MIC, minimum inhibitory concentration; PGP, plant growth promoting.

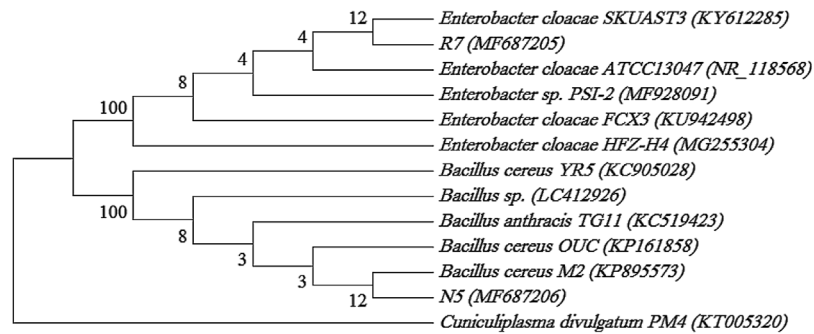


Fig. 5. Phylogenetic tree constructed using 16S rRNA gene sequences, available in the GenBank database, employing the Neighbour-joining method. Bootstrap values based on 1000 replications were listed as percentages at the nodes. The scale bar indicates genetic distance. The GenBank accession number is given in parentheses for each bacterium. The bacterium *Cuniculiplasma divulgatum* was considered as outgroup.

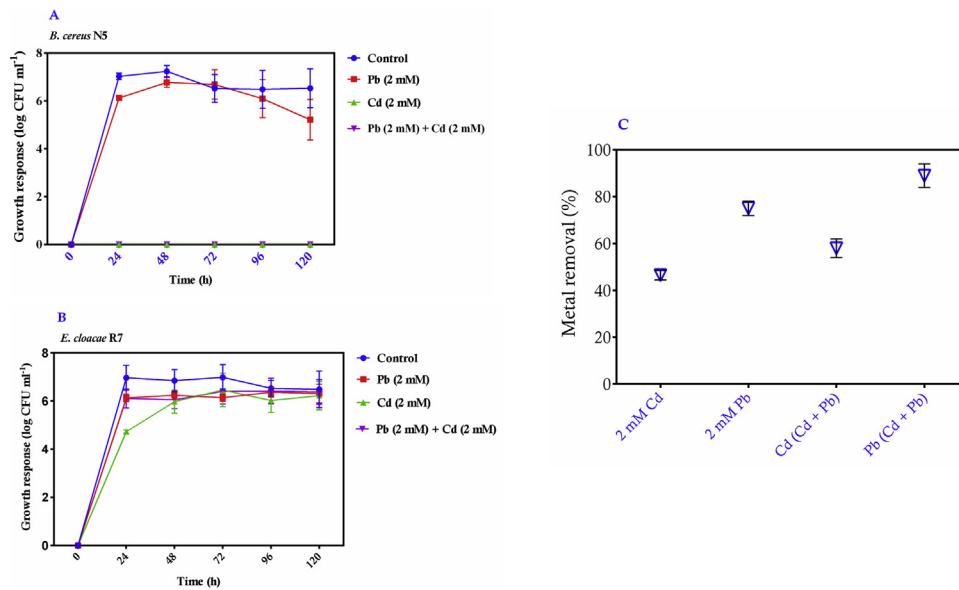


Fig. 6. Comparative growth responses of endophytic strain *B. cereus* N5 (A) and rhizosphere strain *E. cloacae* R7 (B) grown in nutrient broth (NB) with or without (control) Cd (2 mM), Pb (2 mM), and Cd + Pb with the time course and the Cd and Pb removal ability of *E. cloacae* R7 in NB medium supplemented with Cd (2 mM), Pb (2 mM), and Cd + Pb after 48 h (C). Data are the mean of three replications with SE.

areas. Under heavy metal stress, soil microorganisms, including bacteria, have several toxicity resistance mechanisms [12]. Hence, it was expected that maize plants irrigated with industrial and municipal wastewater could harbor heavy metal-resistant bacteria. It has been known that the population of endophytic and rhizospheric bacteria are affected by biological and non-biological agents such as heavy metal stress [28]. In other words, the bacteria surviving in these environments are resistant to environmental stresses, including heavy metal stress. On the other hand, recent advances in research on the interactions between plant and bacteria have shown that plants are able to form their own rhizosphere and endorhiza microbiome (selective effect on entry of bacteria into rhizosphere and endorhiza) [38]. Plants under various environmental stresses, including heavy metals, absorb bacteria that are resistant to stress and can help plant establishment under such conditions [4].

In confirmation of our results, in previous studies, heavy metal-resistant bacteria have been isolated from heavy metals-contaminated environments [39]. For example, in a previous study [40], a total of 22 bacterial isolates were isolated from the rhizosphere of cauliflower plant grown in heavy metals-contaminated environment. These

isolates were able to grow in the presence of toxic metals (concentration of 100 mg L^{-1}) such as Cd, Cr, Cu, Pb, and Ni. These bacterial isolates showed a various level of tolerance to each of five test metals during growth in their culture medium. Bestawy et al. [41] also isolated eight bacterial isolates from soils irrigated with wastewater. These isolates were able to withstand 275 mg L^{-1} Cu, 320 mg L^{-1} Cd, 140 mg L^{-1} Co, and 29 mg L^{-1} Cr.

In this study, the resistance of rhizospheric bacteria to heavy metals was higher than that of endophytic bacteria. It is known that endophytic bacteria are less resistant to environmental stress due to the fact that they are not in direct contact with the outside environment [42]. This could be a possible cause for higher resistance of the rhizospheric bacteria to heavy metals than endophytic isolates. Guo et al. [30] also demonstrated that the endophytic bacteria isolated from the leaf of *Solanum nigrum* L. plant grown in heavy metals (Cd)-contaminated soils had lower resistance to heavy metals than endophytic bacteria isolated from the root of the plant. According to these researchers, this was due to the higher concentration of Cd in the root relative to the leaf of this plant and as a result the greater resistance of the isolates isolated from the root to heavy metals relative to the isolates

isolated from the leaves. In addition, the selective pressure of plants has the maximum effect on the population of bacteria [43] and on selection of the bacteria with specific traits.

In general, endophytic bacteria may be of particular importance because they have relatively good protection from a competitive environment and high stresses in soil [44]. In previous studies, heavy metal-resistant endophytic bacteria were also isolated from plants. As an example, in a study [30], the endophytic isolates, isolated from leaf of *Solanum nigrum* L. plant grown in heavy metals-contaminated soils, showed resistance to all heavy metals Cd, Pb, Zn, Cu, and Cr.

In this study, after Co, the most toxic element for both rhizosphere isolates and endophytic ones was Cd. In previous studies, Cd was also reported as the most toxic element for bacteria. In the study performed by Guo et al. [30], the toxicity of metals for *Bacillus* sp. L14 was reported as Cd (II) > Pb (II) > Zn (II) > Cu (II) > Cr (VI). In another study [45], six bacterial isolates were isolated from the rhizosphere of plants grown around mine. Heavy metals toxicity for these isolates was reported as Cd²⁺ > Cu²⁺ > Zn²⁺ > Pb²⁺.

In this study, the endophytic bacterial isolates were not resistant to Cd compared to rhizosphere isolates. This is one of the interesting results of this research, which requires more study in the future. The question raised by this result can be that if one of the origins of endophytic bacteria is rhizospheric bacteria, why these bacterial isolates are not resistant to Cd or to low concentrations of Cd. Addressing this question requires further research in the future. In general, the bacterial isolates isolated in this study had an acceptable potential for resistance to heavy metals. The ability to grow even at higher concentrations of metals in many microorganisms, including bacteria, has been reported in previous studies, which can be the result of intrinsic or induced mechanisms, as well as other environmental factors (i.e., pH, metal speciation, reduction, etc.) which reduce the toxicity of metals [46–48].

In addition to resistance to heavy metals, the endophytic and rhizosphere bacterial isolates of this study also showed a remarkable resistance to salinity. In general, there are few reports about halophilic bacteria resistant to heavy metals [49,50]. Recently, Jiang et al. [45] studied the ability to tolerate salinity by six heavy metal-resistant bacterial isolates. They showed that all six isolates (*Chryseobacterium indoltheticum*, *Cupriavidus oxalaticus*, *Pseudomonas helmanticensis*, *Bacillus mycoides*, *B. almalaya*, and *Acinetobacter tjernbergiae*) were resistant to 1–7% salinity [45]. The results of this assessment showed that these bacteria have the potential to be used in salinity- and heavy metal-polluted areas.

Endophytic and rhizospheric bacterial isolates of this study showed multiple PGP traits such as IAA production, ACC deaminase, and siderophore and phosphate solubilization. In previous studies, endophytic and rhizospheric bacteria resistant to heavy metals also showed these PGP characteristics [12,28]. This indicates that heavy metal toxicity has not been able to prevent the production of such PGP properties in bacteria associated with heavy metal-stressed plants (e.g., maize plants irrigated with wastewater in this study). The ability to produce IAA among these isolates was very variable. For example, a high percentage of endophytic and rhizospheric bacteria produced IAA. It has been reported that 80% of microorganisms isolated from rhizosphere of different plants had the ability to produce IAA as secondary metabolites [51]. Since IAA is a plant hormone that has no specific role in bacterial cells, it can be assumed that IAA production can improve the ability of these isolates to interact with plants [52,53]. Previous studies have reported the role of this hormone in helping bacteria to become plant endophytes [54]. The results of this study showed that the abundance of IAA-producing endophytic isolates was higher than that of the IAA-producing rhizosphere isolates. This conclusion is consistent with the results of a previous study [55]. Since the first stage of bacterial infiltration into the root of the

plant involves the adherence of the bacteria on the epidermal cell of the root surface and mainly on the base area of the exit of root hairs, it is possible that IAA-producing isolates can, by increasing the root system, colonize the plant root better than other isolates. The bacterial IAA loosens the cell wall of the plant and thus increases the amount of root secretions that cause a large amount of nutrients to grow rhizospheric bacteria [53]. Additionally, successful entry of endophytes into the host plant is through root tips and cracks made at the outlet side of lateral roots [56]. Since IAA promotes the development of the root system of the host plant, the IAA-producing isolates can increase the ability of these isolates to colonize the plant [51].

In the research, a high percentage of bacterial isolates produced ACC deaminase. Earlier studies have reported that beneficial bacteria bind to the surface of roots or seeds, and can absorb some of the ACC secreted by the plant and degrade it by the ACC deaminase enzyme [57]. It is believed that the bacteria that produce this enzyme at the root of the plant act as a reservoir for ACC, which reduces the level of exogenous ethylene, thereby reducing the stress caused by heavy metals and increasing the elongation of the root [58]. The ability to produce siderophore is one of the features that make microorganisms more successful in competing with other microorganisms in environment, facilitating plant-bacteria communication and helping root colonization [59]. This can be a reason for the greater abundance of siderophore-producing bacteria in this study. The high abundance of IAA- and ACC deaminase-producing isolates founded in this study led to the conclusion that host plants are a selective agent for specific bacterial species that select partners from soil or seeds. The ability of such bacteria to colonize both external and internal plant tissues is a desirable feature for inoculation of seeds because such bacteria have a greater chance of having an effect on host growth [52]. The results of this study also showed that most of the bacterial isolates resistant to heavy metals had multiple PGP traits (Fig.3). These bacteria may be used to enhance maize plant growth and resistance under heavy metal stress conditions. Each of these PGP characteristics can somehow reduce the toxicity of heavy metals to the plant [12]. In this study, the best bacterial strains belonged to the genera *Enterobacter* and *Bacillus*. In previous studies, such genera have been isolated from corn roots with a potential for plant growth promotion [60,61]. Resistance to heavy metals, including Pb, in genus *Bacillus* has been reported in previous studies [40,62].

In the early evaluation of the endophytic strain *B. cereus* N5 in this study, it was found that strain N5 was a cadmium-sensitive bacterium in NA-medium supplemented with Cd; in this assessment, it was not also able to grow in the presence of Cd in liquid medium. As described above, none of the endophytic bacterial isolates were resistant to Cd. But in a previous study, *B. cereus* KTSMBNL 43 was recognized as an effective bacterium at the removal of Cd (up to 82%) in a medium containing 200 mg L⁻¹ Cd [36]. But in our study, *B. cereus* N5 was not able to grow in the medium containing Cd. According to Guo et al. [30], a possible cause of the bacterium's lack of resistance to Cd can be due to the lower concentration of Cd in the root tissue. In addition, rhizosphere bacteria are in direct contact with heavy metals compared with endophytic bacteria. This may be another possible cause for greater resistance of rhizospheric bacteria to Cd. However, more research is needed to find out why endophytic bacterial isolates of this study were not resistant to Cd. Previous studies have also shown that Cd is more toxic to bacteria than Pb [40,45]. However, in this study, some rhizosphere bacteria including strain *E. cloacae* R7, showed a remarkable resistance to Cd (2 mM). In a previous study [36], bacteria that were able to grow at a concentration of 200 mg L⁻¹ Cd were introduced as Cd-resistant bacteria. In another study, *E. cloacae*, isolated from soils

contaminated to Cd, could withstand a concentration of 300 mg L⁻¹ Cd [63]. As shown in Fig. 6B, the rhizosphere strain *E. cloacae* R7 in the presence of Cd and Pb simultaneously had a better growth than its growth in the presence of these metals separately. This finding is consistent with the results of a previous study [64]. Previous research has also shown that such bacteria (e.g., *Enterobacter*) had the ability to withstand the high concentrations of heavy metals [41,63,65].

In a study by Oves et al. [40], adsorption of heavy metals by *Bacillus* biomass had a similar trend (Ni > Cu > Pb > Cr > Cd). In this study, the percentage of Pb removal was higher than that of Cd. There are numerous reports on the use of living bacterial cells for bio-refining heavy metals-contaminated water and soils [36,66,67]. Among them, numerous studies have shown that bacteria belonging to the genus *Pseudomonas* and *Bacillus* have successfully been used as metal bio-absorbents due to their high binding to heavy metals [67–69]. In another study by Lu, et al. [70], *Enterobacter* sp. J1, which was isolated from industrial wastewater, was able to grow in media containing 1500 mg L⁻¹ Pb, 500 mg L⁻¹ Cu, 300 mg L⁻¹ Cd, 750 mg L⁻¹ Zn, and 300 mg L⁻¹ Ni. The biomass of this strain was also able to remove 75 and 90% Pb and Cd, respectively [70]. But in this assessment, *E. cloacae* R7 removed a lower percentage of Cd. Past research has shown that the adsorption of metal ions by microbial biomass depends mainly on the active agent groups in the active bacterial cells and on the physiological conditions of the solution [40]. It has been reported that the rate of growth and the amount of biomass production are directly related to the absorption of metal by bacteria [30]. Since Cd had a higher toxicity to bacteria than Pb (reduction of growth and biomass of bacteria), the reduction in the percentage of Cd metal removal than Pb by *E. cloacae* R7 can be justified in this assessment. Previous studies have shown that the growth and biomass of bacteria in the presence of multi-metals are higher than their growth in the presence of these metals separately [64]. This finding could be the reason why metal removal by strain R7 in a composite state might be more than removing the metal in a separate state. Various mechanisms of resistance of bacteria to heavy metals in various bacteria have been identified [12]. Many researchers have reported that the bacterial cell wall has several carboxylic, hydroxyl, phosphate, amide and sulfate groups, which cause metal ions to bind to the wall of the bacteria [71].

Root colonization is a key factor in the efficiency of bacterial inoculum that can be effective at stimulating plant growth and biological control activity [72]. *E. cloacae* R7 also showed the higher population size on maize plant roots under metal stress compared to non-stress conditions. It was proven that the one of the plant responses to metal stress is to alter root morphology such as increased surface area, due to the formation of root hairs and lateral roots [73]. In addition, it is well found that beneficial bacteria, under stress conditions, such as salinity, drought, lack of a nutrient, etc., can exert a positive effect on the plant compared to non-stress conditions [2]. Therefore, heavy metal stress-mediated increase of root surface area and higher production of IAA by strain R7 under Cd and Pb stress can be reason for increasing population size of strain R7 on root surface and inside roots of maize plant in the presence of Cd and Pb, because bacterial IAA, by increasing root system, can provide more active sites to colonization of beneficial bacteria [53].

In general, the results of this assessment showed that strain R7 also had the potential to enter and colonize maize plant roots in the presence and the absence of Cd and Pb, in addition to colonizing the root surface or rhizosphere. In other words, strain R7 is a facultative endophyte. Several studies have reported that the origin of endophytic bacteria can also be from population of rhizospheric bacteria [2,74]. Researchers have shown that the genera *Pseudomonas*, *Enterobacter*, and *Bacillus* were also isolated

as common endophytes from maize plant and other plants [33]. In previous studies, we also observed that rhizospheric bacteria can also become an endophyte [19,35]. It is known that the entry of bacteria into the plant can be through cracks in the base of the lateral roots and in epidermis [75]. In addition, it has been shown that the infiltration of bacteria into the plant is not necessarily an active mechanism, and therefore all rhizospheric bacteria can be expected to become an endophyte at one stage of their life [4].

5. Conclusions

The results of this study showed that rhizosphere and endorhiza of maize plant irrigated with industrial and municipal wastewater harbor heavy metals-resistant bacteria with multiple beneficial effects and various ability of production of PGP traits. Both endophytic isolates and rhizosphere ones showed remarkable resistance to salinity (7% NaCl). Compared to endophytic isolates, rhizosphere isolates had greater resistance to heavy metals. Since the use of industrial and municipal wastewaters in agriculture in recent years has become commonplace, the use of the salinity- and heavy metal-resistant PGP bacteria may alleviate the stresses originated from the use of the wastewater in maize plant and may also reduce the accumulation of heavy metals in plants irrigated with the wastewater. For example, *E. cloacae* R7 identified in this study, due to the production of higher biomass and colonization of the plant in the presence of heavy metals, being resistant to several heavy metals and salinity, and having multiple PGP traits, can be a cost-effective and environmentally friendly bio-adsorbent for removing heavy metals (e.g., Cd and Pb) in aqueous media. Further assay of the strain on soil-plant (maize) system under greenhouse and field conditions is needed to uncover its efficacy as an effective beneficial bacterium in salinity- and heavy metal-contaminated soils.

Author contributions

All authors had the same contribution.

Conflict of interest

The authors (Hassan Etesami, Hossein Ali Alikhani, Motahhareh Abedinzadeh) declare that they have no conflict of interest

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References

- [1] R.P. Ryan, K. Germaine, A. Franks, D.J. Ryan, D.N. Dowling, Bacterial endophytes: recent developments and applications, *FEMS Microbiol. Lett.* 278 (2008) 1–9.
- [2] H. Etesami, D.K. Maheshwari, Use of plant growth promoting rhizobacteria (PGPRs) with multiple plant growth promoting traits in stress agriculture: action mechanisms and future prospects, *Ecotoxicol. Environ. Saf.* 156 (2018) 225–246.
- [3] J.W. Kloepper, R. Lifshitz, R.M. Zablottowicz, Free-living bacterial inocula for enhancing crop productivity, *Trend. Biotechnol.* 7 (1989) 39–44.
- [4] P.R. Hardoim, L.S. van Overbeek, J.D. van Elsas, Properties of bacterial endophytes and their proposed role in plant growth, *Trend. Microbiol.* 16 (2008) 463–471.
- [5] R.L. Berendsen, C.M.J. Pieterse, P.A.H.M. Bakker, The rhizosphere microbiome and plant health, *Trend. Plant Sci.* 17 (2012) 478–486.
- [6] P.R. Hardoim, L.S. van Overbeek, J.D. van Elsas, Properties of bacterial endophytes and their proposed role in plant growth, *Trend Microbiol.* 16 (2008) 463–471.
- [7] R. Rodriguez, R. Redman, More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis, *J. Exp. Bot.* 59 (2008) 1109–1114.

- [8] C. Leifert, W.M. Waites, J.R. Nicholas, Bacterial contaminants of micropropagated plant cultures, *J. Appl. Bacteriol.* 67 (1989) 353–361.
- [9] G. Patel, Effect of Waste Irrigation on Heavy Metal Accumulation in Soil and its Removal by Plants, (2013).
- [10] R.K. Rattan, S.P. Datta, P.K. Chhonkar, K. Suribabu, A.K. Singh, Long-term impact of irrigation with sewage effluents on heavy metal content in soils, crops and groundwater—a case study, *Agric. Ecosyst. Environ.* 109 (2005) 310–322.
- [11] M.R. Noll, Trace elements in terrestrial environments: biogeochemistry, bioavailability, and risks of metals, *J. Environ. Qual.* 32 (2003) 374.
- [12] H. Etesami, Bacterial mediated alleviation of heavy metal stress and decreased accumulation of metals in plant tissues: mechanisms and future prospects, *Ecotoxicol. Environ. Saf.* 147 (2018) 175–191.
- [13] P.A. Wani, M.S. Khan, A. Zaidi, Chromium-reducing and plant growth-promoting *Mesorhizobium* improves chickpea growth in chromium-amended soil, *Biotech. Lett.* 30 (2008) 159–163.
- [14] M. Ahemad, M.S. Khan, Effect of fungicides on plant growth promoting activities of phosphate solubilizing *Pseudomonas putida* isolated from mustard (*Brassica campestris*) rhizosphere, *Chemosphere* 86 (2012) 945–950.
- [15] A. Giongo, A. Ambrosini, L.K. Vargas, J.R.J. Freire, M.H. Bodanese-Zanettini, L.M. P. Passaglia, Evaluation of genetic diversity of bradyrhizobia strains nodulating soybean [*Glycine max* (L.) Merrill] isolated from South Brazilian fields, *Appl. Soil Ecol.* 38 (2008) 261–269.
- [16] C. Sánchez-Porro, R. Rafael, N. Soto-Ramírez, M.C. Márquez, R. Montalvo-Rodríguez, A. Ventosa, Description of *Kushneria aurantia* gen. nov., sp. nov., a novel member of the family Halomonadaceae, and a proposal for reclassification of *Halomonas marisflavi* as *Kushneria marisflavi* comb. nov., of *Halomonas indaliniina* as *Kushneria indaliniina* comb. nov. and of *Halomonas avicenniae* as *Kushneria avicenniae* comb. nov., *Int. J. Syst. Evol. Microbiol.* 59 (2009) 397–405.
- [17] J.-H. Yoon, S.H. Choi, K.-C. Lee, Y.H. Kho, K.H. Kang, Y.-H. Park, *Halomonas marisflavae* sp. nov., a halophilic bacterium isolated from the Yellow Sea in Korea, *Int. J. Syst. Evol. Microbiol.* 51 (2001) 1171–1177.
- [18] J.R. Okalebo, K.W. Gathua, P.L. Woome, Laboratory Methods of Soil and Plant Analysis: a Working Manual, second edition, Sacred Africa, Nairobi, 2002.
- [19] H. Etesami, H.M. Hosseini, H.A. Alikhani, L. Mohammadi, Bacterial biosynthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase and indole-3-acetic acid (IAA) as endophytic preferential selection traits by rice plant seedlings, *J. Plant Growth Regul.* 33 (2014) 654–670.
- [20] S. Chatterjee, G.B. Sau, S.K. Mukherjee, Plant growth promotion by a hexavalent chromium reducing bacterial strain, *Celluloseimicrobiology cellulans KUCr3*, *World J. Microbiol. Biotechnol.* 25 (2009) 1829–1836.
- [21] F. Noori, H. Etesami, H. Najafi Zarini, N.A. Khoshkholgh-Sima, G. Hosseini Salekdeh, F. Alishahi, Mining alfalfa (*Medicago sativa* L.) nodules for salinity tolerant non-rhizobial bacteria to improve growth of alfalfa under salinity stress, *Ecotoxicol. Environ. Saf.* 162 (2018) 129–138.
- [22] C.L. Patten, B.R. Glick, Bacterial biosynthesis of indole-3-acetic acid, *Can. J. Microbiol.* 42 (1996) 207–220.
- [23] B. Schwyn, J.B. Neilands, Universal chemical assay for the detection and determination of siderophores, *Biochem. Anal. Biochem.* 160 (1987) 47–56.
- [24] J.I. Sperber, Solution of apatite by soil microorganisms producing organic acids, *Crop Past. Sci.* 9 (1958) 782–787.
- [25] D.M. Penrose, B.R. Glick, Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria, *Physiol. Plant.* 118 (2003) 10–15.
- [26] U. Edwards, T. Rogall, H. Blocker, M. Emde, E.C. Bottger, Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA, *Nucleic Acids Res.* 17 (1989) 7843–7853.
- [27] H. Etesami, H.A. Alikhani, Co-inoculation with endophytic and rhizosphere bacteria allows reduced application rates of N-fertilizer for rice plant, *Rhizosphere* 2 (2016) 5–12.
- [28] M. Rajkumar, N. Ae, H. Freitas, Endophytic bacteria and their potential to enhance heavy metal phytoextraction, *Chemosphere* 77 (2009) 153–160.
- [29] M. Zubair, M. Shakir, Q. Ali, N. Rani, N. Fatima, S. Farooq, S. Shafiq, N. Kanwal, F. Ali, I.A. Nasir, Rhizobacteria and phytoremediation of heavy metals, *Environ. Technol. Rev.* 5 (2016) 112–119.
- [30] H. Guo, S. Luo, L. Chen, X. Xiao, Q. Xi, W. Wei, G. Zeng, C. Liu, Y. Wan, J. Chen, Bioremediation of heavy metals by growing hyperaccumulaor endophytic bacterium *Bacillus* sp. L14, *Bioresour. Technol.* 101 (2010) 8599–8605.
- [31] C. Leifert, W.M. Waites, J.R. Nicholas, Bacterial contaminants of micropropagated plant cultures, *J. Appl. Microbiol.* 67 (1989) 353–361.
- [32] M. Rosenblueth, E. Martinez Romero, *Rhizobium etli* maize populations and their competitiveness for root colonization, *Arch. Microbiol. Res.* 181 (2004) 337–344.
- [33] J. Hallmann, Bacterial endophytes in agricultural crops, *Can. J. Microbiol.* 43 (1997) 895–914.
- [34] D.Y. Kobayashi, J.D. Palumbo, Bacterial endophytes and their effects on plants and uses in agriculture, in: C.W. Bacon, J.F. White (Eds.), *Microbial Endophytes*, Marcel Dekker, New York, USA, 2000, pp. 199–233.
- [35] H. Etesami, H.A. Alikhani, Rhizosphere and endorhiza of oilseed rape (*Brassica napus* L.) plant harbor bacteria with multifaceted beneficial effects, *Biol. Control* 94 (2016) 11–24.
- [36] P. Arivalagan, D. Singaraj, V. Haridass, T. Kaliannan, Removal of cadmium from aqueous solution by batch studies using *Bacillus cereus*, *Ecol. Eng.* 71 (2014) 728–735.
- [37] M. Rajkumar, N. Ae, M.N. Prasad, H. Freitas, Potential of siderophore-producing bacteria for improving heavy metal phytoextraction, *Trend Biotechnol.* 28 (2010) 142–149.
- [38] C.M.J. Pieterse, C. Zamioudis, R.L. Berendsen, D.M. Weller, S.C.M. Van Wees, P.A. H.M. Bakker, Induced systemic resistance by beneficial microbes, *Ann. Rev. Phytopathol.* 52 (2014) 347–375.
- [39] S. Abdelkrim, S.H. Jebara, O. Saadani, M. Chiboub, G. Abid, K. Mannai, M. Jebara, Heavy metal accumulation in *Lathyrus sativus* growing in contaminated soils and identification of symbiotic resistant bacteria, *Arch. Microbiol.* (2018) 1–15.
- [40] M. Oves, M.S. Khan, A. Zaidi, Biosorption of heavy metals by *Bacillus thuringiensis* strain OSM29 originating from industrial effluent contaminated north Indian soil, *Saudi J. Biol. Sci.* 20 (2013) 121–129.
- [41] E.E. Bestawy, S. Helmy, H. Hussien, M. Fahmy, R. Amer, Bioremediation of heavy metal-contaminated effluent using optimized activated sludge bacteria, *Appl. Water Sci.* 3 (2013) 181–192.
- [42] J. Hallmann, A. Quadt-Hallmann, W.F. Mahaffee, J.W. Kloepper, Bacterial endophytes in agricultural crops, *Can. J. Microbiol.* 43 (1997) 895–914.
- [43] S.D. Siciliano, N. Fortin, A. Mihoc, G. Wisse, S. Labelle, D. Beaumier, D. Ouellette, R. Roy, L.G. Whyte, M.K. Banks, Selection of specific endophytic bacterial genotypes by plants in response to soil contamination, *Appl. Environ. Microbiol.* 67 (2001) 2469–2475.
- [44] A.V. Sturz, J. Nowak, Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops, *Appl. Soil Ecol.* 15 (2000) 183–190.
- [45] J. Jiang, C. Pan, A. Xiao, X. Yang, G. Zhang, Isolation, identification, and environmental adaptability of heavy-metal-resistant bacteria from ramie rhizosphere soil around mine refinery, *3 Biotech* 7 (2017) 5.
- [46] X. Xiao, S. Luo, G. Zeng, W. Wei, Y. Wan, L. Chen, H. Guo, Z. Cao, L. Yang, J. Chen, Biosorption of cadmium by endophytic fungus (EF) *Microsphaeropsis* sp. LSE10 isolated from cadmium hyperaccumulator *Solanum nigrum* L., *Bioresour. Technol.* 101 (2010) 1668–1674.
- [47] A. Leedjäv, A. Ivask, M. Virta, Interplay of different transporters in the mediation of divalent heavy metal resistance in *Pseudomonas putida* KT2440, *J. Bacteriol.* 190 (2008) 2680–2689.
- [48] A.I. Zouboulis, M.X. Loukidou, K.A. Matis, Biosorption of toxic metals from aqueous solutions by bacteria strains isolated from metal-polluted soils, *Process Biochem.* 39 (2004) 909–916.
- [49] L.I.U. Ai-min, Study on the resistance of a halotolerant and alkalotolerant bacterium, *J. Anhui Norm. Univ. (Nat. Sci.)* 2 (2005) 020.
- [50] D.M. Voica, L. Bartha, H.L. Banciu, A. Oren, Heavy metal resistance in halophilic Bacteria and Archaea, *FEMS Microbiol. Lett.* 363 (2016).
- [51] C.L. Patten, B.R. Glick, Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system, *Appl. Environ. Microbiol.* 68 (2002) 3795–3801.
- [52] H. Etesami, H.A. Alikhani, H.M. Hosseini, Indole-3-acetic acid (IAA) production trait, a useful screening to select endophytic and rhizosphere competent bacteria for rice growth promoting agents, *MethodsX* 2 (2015) 72–78.
- [53] H. Etesami, H.A. Alikhani, H.M. Hosseini, Indole-3-acetic acid and 1-aminocyclopropane-1-carboxylate deaminase: bacterial traits required in rhizosphere, rhizoplane and/or endophytic competence by beneficial bacteria, *Bacterial Metabolites in Sustainable Agroecosystem*, Springer, 2015, pp. 183–258.
- [54] S.C. Verma, J.K. Ladha, A.K. Tripathi, Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice, *J. Biotechnol.* 91 (2001) 127–141.
- [55] R. Mendes, A.A. Pizzirani-Kleiner, W.L. Araujo, J.M. Raaijmakers, Diversity of cultivated endophytic bacteria from sugarcane: genetic and biochemical characterization of *Burkholderia cepacia* complex isolates, *Appl. Environ. Microbiol.* 73 (2007) 7259–7267.
- [56] F. Chi, S.-H. Shen, H.-P. Cheng, Y.-X. Jing, Y.G. Yanni, F.B. Dazzo, Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology, *Appl. Environ. Microbiol.* 71 (2005) 7271–7278.
- [57] B.R. Glick, Bacteria with ACC deaminase can promote plant growth and help to feed the world, *Microbiol. Res. (Pavia)* 169 (2014) 30–39.
- [58] B.R. Glick, Z. Cheng, J. Czarny, J. Duan, Promotion of plant growth by ACC deaminase-producing soil bacteria, *Eur. J. Plant Pathol.* 119 (2007) 329–339.
- [59] I. Loaces, L. Ferrando, A.F. Scavino, Dynamics, diversity and function of endophytic siderophore-producing bacteria in rice, *Microbiol. Ecol.* 61 (2011) 606–618.
- [60] S. Liu, J. Yang, L. Ma, C. Zhang, Y. He, L. Pu, Effect of *Bacillus amyloliquefaciens* B9601-Y2 on growth and yield promotion of maize, *J. Maize Sci.* 18 (2010) 78–82.
- [61] M.A. Abiala, A.C. Odebo, Rhizospheric *Enterobacter* enhanced maize seedling health and growth, *Biocontrol Sci. Technol.* 25 (2015) 359–372.
- [62] S. Luo, X. Xiao, Q. Xi, Y. Wan, L. Chen, G. Zeng, C. Liu, H. Guo, J. Chen, Enhancement of cadmium bioremediation by endophytic bacterium *Bacillus* sp. L14 using industrially used metabolic inhibitors (DCC or DNP), *J. Hazard. Mater.* 190 (2011) 1079–1082.
- [63] L. Xiaoyan, M. Renxiang, C. Zhaoyun, Isolation and cadmium adsorption mechanisms of cadmium-resistant bacteria strains, *J. Agro Environ. Sci.* 34 (2015) 1700–1706.
- [64] P. Yuanyuan, C. Wenli, H. Qiaoyun, Isolation, identification and 16S rDNA sequence analysis of a strain with resistance to Cu and Cd, *Microbiol. Chin.* 32 (2005) 68–72.
- [65] J. Suriya, S. Bharathiraja, R. Rajasekaran, Biosorption of heavy metals by biomass of *Enterobacter cloacae* isolated from metal-polluted soils, *Int. J. ChemTech Res.* 5 (2013) 1329–1338.
- [66] A. Rathinam, B. Maharshi, S.K. Janardhanan, R.R. Jonnalagadda, B.U. Nair, Biosorption of cadmium metal ion from simulated wastewaters using *Hypnea valentiae* biomass: a kinetic and thermodynamic study, *Bioresour. Technol.* 101 (2010) 1466–1470.

- [67] X.-x. Zeng, J.-x. Tang, X.-d. Liu, P. Jiang, Isolation, identification and characterization of cadmium-resistant *Pseudomonas aeruginosa* strain E 1, J. Cent. South Univ. Technol. 16 (2009) 416–421.
- [68] J.-H. Joo, S.H.A. Hassan, S.-E. Oh, Comparative study of biosorption of Zn²⁺ by *Pseudomonas aeruginosa* and *Bacillus cereus*, Inte. Biodeteriorat. Biodegrad. 64 (2010) 734–741.
- [69] F. Huang, Z. Dang, C.-L. Guo, G.-N. Lu, R.R. Gu, H.-J. Liu, H. Zhang, Biosorption of Cd (II) by live and dead cells of *Bacillus cereus* RC-1 isolated from cadmium-contaminated soil, Colloids Surf. B Biointerfaces 107 (2013) 11–18.
- [70] W.-B. Lu, J.-J. Shi, C.-H. Wang, J.-S. Chang, Biosorption of lead, copper and cadmium by an indigenous isolate *Enterobacter* sp. J1 possessing high heavy-metal resistance, J. Hazard Mater. 134 (2006) 80–86.
- [71] K. Vijayaraghavan, Y.-S. Yun, Bacterial biosorbents and biosorption, Biotechnol. Adv. 26 (2008) 266–291.
- [72] H.S.A. Silva, R.d.S. Romeiro, A. Munteer, Development of a root colonization bioassay for rapid screening of rhizobacteria for potential biocontrol agents, J. Phytopathol. 151 (2003) 42–46.
- [73] A. Vitti, M. Nuzzaci, A. Scopa, G. Tataranni, I. Tamburrino, A. Sofo, Hormonal response and root architecture in *Arabidopsis thaliana* subjected to heavy metals, Inte. J. Plant Biol. 5 (2014).
- [74] M. Elvira-Recuenco, J.W. Van Vuurde, Natural incidence of endophytic bacteria in pea cultivars under field conditions, Can. J. Microbiol. 46 (2000) 1036–1041.
- [75] N. Garg, Symbiotic nitrogen fixation in legume nodules: process and signaling: a review, Sustainable Agriculture, Springer, 2009, pp. 519–531.