Assessment of the plant growth promotion abilities of six bacterial isolates using Zea mays as indicator plant

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ABSTRACT

Zea mays, one of the most important cereals worldwide, is a plant not only with food and energy value, but also with phytoremediation potential. The use of plant growth promoting (PGP) rhizobacteria may constitute a biological alternative to increase crop yield and plant resistance to degraded environments. In search for PGP rhizobacteria strains, 6 bacterial isolates were isolated from a metal contaminated site, screened in vitro for their PGP characteristics and their effects on the growth of Z. mays were assessed. Isolates were identified as 3A101, ECP37, corresponding to Chryseobacterium palustre and Chrysobacterium humi, and 1ZP4, EC15, EC30 and 1C2, corresponding to strains within the genera Sphingobacterium, Bacillus, Achromobacter, and Ralstonia, respectively. All the bacterial isolates were shown to produce indole acetic acid, hydrogen cyanide and ammonia when tested in vitro for their plant growth promoting abilities, but only isolates 1C2, 1ZP4 and ECP37 have shown siderophore production. Their further application in a greenhouse experiment using Z. mays indicated that plant traits such as root and shoot elongation and biomass production, and nutrient status, namely N and P levels, were influenced by the inoculation, with plants inoculated with 1C2 generally outperforming the other treatments. Two other bacterial isolates, 1ZP4 and ECP37 also led to increased plant growth in the greenhouse. These 3 species, corresponding to strains within the genera Ralstonia (1C2), Sphingobacterium (1ZP4), and one strain identified as C. humi (ECP37) can thus be potential agents to increase crop yield in maize plants.

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1. Introduction

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots (Ahmad et al., 2008). The enhancement of crop plant growth using PGPR is documented (Reed and Glick, 2004) and these organisms have been used to reduce plant stress associated with phytoremediation strategies for metal contaminated soils (Reed and Glick, 2005). PGPR enhance plant growth through various forms, such as: (i) reducing ethylene production, allowing plants to develop longer roots and better establish during early stages of growth, due to the synthesis of 1-aminoacyclopropane-1-carboxylate (ACC) deaminase which modulates the level of ethylene by hydrolyzing ACC, a precursor of ethylene, in ammonia and α-ketobutyrate (Glick et al., 1998); (ii) enhancing asymbiotic nitrogen fixation (Khan, 2005) or indirectly affecting symbiotic N2 fixation, nodulation or nodule occupancy (Fuhrmann and Wollum, 1989); (iii) producing or changing the concentration of plant growth regulators like indole acetic acid (IAA) (Ahmad et al., 2008); (iv) raising the solubilisation of nutrients with consequent increase in the supply of bioavailable phosphorous and other trace elements for plant uptake (Glick, 1995); (v) production of phytohormones such as auxins, cytokinins and gibberelins (Glick, 1995); and (vi) synthesis of antibiotic and other pathogen-depressing substances such as siderophores, cyanide and chelating agents that protect plants from diseases (Kamnev and Lelie, 2000). These organisms can also increase plant tolerance to flooding (Grichko and Glick, 2001), salt stress (Mayak et al., 2004a) and water deprivation (Mayak et al., 2004b).

Plant growth promoting bacteria are not only significant from an agricultural point of view, but may also play an important role in soil remediation strategies, not only by enhancing growth and successful establishment of plants in polluted soils, but also by increasing the availability of contaminants, as reported for heavy metals, namely Zn and Ni, in Thlaspi caerulescens (Whiting et al., 2001) and in Alyssum murale and Thlaspi goessingense

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2. Material and methods

2.1. Bacteria isolation and characterization

Selected bacterial species were indigenous to a metal contaminated site. The site has a long history of metal contamination, due to the industrial activity in the surrounding area. Despite the high presence of metals – average levels of 835 mg Pb kg\(^{-1}\), 66 mg Hg kg\(^{-1}\), 26 mg Cr kg\(^{-1}\), 37 mg Ni kg\(^{-1}\), 16,800 mg Fe kg\(^{-1}\) and 3620 mg Zn kg\(^{-1}\) – the area is prolific in vegetation (Marques et al., 2007).

Bacterial isolation was performed from sediments collected at the site. Sediment samples were serially diluted in saline solution (0.85% (w/v) NaCl) and inoculated on trypticase soy agar (TSA; Oxoid) adjusted to pH 5, 6 and 7, using buffer solutions at concentrations of 100 mM at 30°C. Visually different colonies selected on the basis of colony morphology and color were selected and were further purified by subculturing and preserved at –80°C in modified Luria-Bertani broth (MLB) (Tiago et al., 2004), supplemented with 15% (v/v) glycerol. This sampling yielded a total of 320 strains. These strains were grown under heavy metal stress (Zn, Cd and As). Of these, 6 strains were selected based on their metal resistance ability as an additional important value for further applications. Four isolates designated as 1ZP4, EC15, EC30 and 1C2, and 2 isolates identified as ECP37\(^{1}\) and 3A10\(^{1}\) (Pires et al., 2010) were used. Tests listed below were performed on all strains. The pH range for growth was determined in buffered tryptase soy broth (TSB) adjusted at pH 3 for 24 h with 3 ml of phosphate buffer (pH 7.5) with glucose (1%) and tryptophan (1%). After incubation, 2 ml of 5% trichloroacetic acid and 1 ml of 0.5 M CaCl\(_2\) were added. The solution was filtered (Whatman No. 2 of pore size) and to 3 ml of the filtrate 2 ml of salper solution (2 ml 0.5 M FeCl\(_3\) and 98 ml 35% perchloric acid) were added. This mixture was incubated for 30 min at 25°C in the dark. The absorbance of the resulting solution was measured at 535 nm with a Shimadzu UV-1603 spectrophotometer.

For assessing the ability to produce NH\(_3\), fresh cultures were inoculated into 10 ml peptone water and incubated for 48–72 h at 30°C; following this, 0.5 ml of Nessler’s reagent were added to each tube and development of yellow to brown color was considered as a positive result for ammonia production (Cappuccino and Sherman, 1992). The screening of hydrogen cyanide production by the bacterial isolates was made by adding nutrient agar with 4.4 g glycine/l and streaking the isolates on this modified agar plates; a Whatman no.1 filter paper soaked in a 2% sodium carbonate in 0.5% picric acid solution was placed on top of each plate and plates were sealed and incubated at 30°C for 4 d after which development of orange to red color indicated HCN production by the isolates (Ahmad et al., 2008). Bacterial isolates were assayed for siderophores production by spot inoculating the isolates (10 µl of 10\(^6\) CFU/ml) on Chrome azurol S agar medium; development of a yellow to orange halo around the bacterial growth after incubation at 30°C for 48–72 h indicated a positive result for siderophore production (Schwyn and Neillands, 1987).

ACC-deaminase activity was assayed according to a modification of the method of Honma and Shimomura (1978) which measures the amount of \(\alpha\)-ketobutyrate produced upon the hydrolysis of ACC. The number of \(\mu\)mol of \(\alpha\)-ketobutyrate produced by this reaction was determined by comparing to a standard curve of \(\alpha\)-ketobutyrate ranging between 0.1 and 1 \(\mu\)mol to which 2 ml of 2,4-dinitrophenylhydrazine (0.2% 2,4-dinitrophenylhydrazine in 2 mol l\(^{-1}\) HCl) was added to each standard, and was then vortexed and incubated at 30°C for 30 min. Color was developed by the addition of 2 ml, 2 mol l\(^{-1}\) NaOH, and the absorbance of the mixture was measured after mixing by using UNICAM HELIOS\(^{1}\) spectrophotometer (Waltham, USA), at 540 nm. For determining ACC-deaminase activity, the bacterial cell pellets were suspended in 5 ml of 0.1 mol l\(^{-1}\) Tris–HCl, pH 7.6, transferred to microcentrifuge tubes and centrifuged at 16,000 rpm for 5 min; the pellets were suspended in 2 ml 0.1 mol l\(^{-1}\) Tris HCl, pH 8.5. Thirty \(\mu\)l of toluene were added to the cell suspension and vortexed. The 30 s and 200 µl of the resulting suspension were placed in a fresh microcentrifuge tube, to which 20 µl of 0.5 mol l\(^{-1}\) ACC were added; samples were vortexed, and incubated at 30°C for 15 min. Following the addition of 1 ml of 0.56 mol l\(^{-1}\) HCl, the mixture was vortexed and centrifuged for 5 min at 16,000 rpm at room temperature and 2 ml of the resulting supernatant were vortexed together with 1 ml of 0.56 mol l\(^{-1}\) HCl, after which 2 ml of 2,4-dinitrophenylhydrazine reagent was added, following vortexing and incubation at 30°C for 30 min. Two ml of 2 mol l\(^{-1}\) NaOH were added and mixed and the absorbance of the mixture was read at 540 nm.

For all the above mentioned tests, sterile nutrient broth or agar were used as a control for bacterial growth.

2.2. IAA production for detection of plant growth promoting ability

IAA production by the bacterial isolates was measured by the method of Wohler (1997). The bacteria were grown overnight on nutrient broth and then collected by centrifugation at 7000 g for 5 min. The bacterial pellet was incubated at 37°C for 24 h with 3 ml of 2 ml of 0.1 mol l\(^{-1}\) Tris–HCl, pH 7.6, transferred to microcentrifuge tubes and centrifuged at 16,000 rpm for 5 min; the pellets were suspended in 2 ml 0.1 mol l\(^{-1}\) Tris HCl, pH 8.5. Thirty µl of toluene were added to the cell suspension and vortexed. The 30 s and 200 µl of the resulting suspension were placed in a fresh microcentrifuge tube, to which 20 µl of 0.5 mol l\(^{-1}\) ACC were added; samples were vortexed, and incubated at 30°C for 15 min. Following the addition of 1 ml of 0.56 mol l\(^{-1}\) HCl, the mixture was vortexed and centrifuged for 5 min at 16,000 rpm at room temperature and 2 ml of the resulting supernatant were vortexed together with 1 ml of 0.56 mol l\(^{-1}\) HCl, after which 2 ml of 2,4-dinitrophenylhydrazine reagent was added, following vortexing and incubation at 30°C for 30 min. Two ml of 2 mol l\(^{-1}\) NaOH were added and mixed and the absorbance of the mixture was read at 540 nm.

For all the above mentioned tests, sterile nutrient broth or agar were used as a control for bacterial growth.

2.3. Zea mays growth – experimental design

Z. mays seeds were surface sterilised with 0.5% (v/v) NaClO for 10 min and were subsequently washed with sterilised deionised water. Seeds were germinated in plastic pots (8 cm diameter) with about 300 g sterilised (120°C for 70 min in two consecutive days) agricultural soil (soil properties are shown in Table 1), in order to ensure that possible observed differences in plant traits were caused only by the applied bacterial treatments. Each pot received

(Abou-Shanab et al., 2003; Idris et al., 2004). Zea mays is a plant with food and energy value (Solomon et al., 2007) and also with phyto remediation potential (Lin et al., 2008; Meers et al., 2010). The aim of this work was to assess the effect of plant growth promoting organisms on the growth of Z. mays. A series of rhizospheric bacterial species were isolated from a metal contaminated site and were screened for their plant growth promotion abilities, assessing IAA, siderophores, hydrogen cyanide, ACC-deaminase activity and ammonia production. Selected species were further inoculated onto Z. mays in order to evaluate their effect on plant growth and biomass production, and on P and N assimilation.
Results are expressed as means ± SD (n = 3); L.O.D. is the method detection limit.

10 seeds. Pots were randomised on the greenhouse, process that was repeated every two weeks during the experiment. After sowing, seedlings were reduced to three per pot; the pots were then inoculated by spraying the soil surface with 10 ml of a solution of each bacterial strain (10^8 CFU/ml) (Vivas et al., 2006) pre-grown in nutrient broth medium for 24–48 h at 28 °C. Ten ml of nutrient broth was also added to the control treatment pots.

The plants were maintained in a controlled growth room (12 h photoperiod, 450 μmol m−2 s−1 photosynthetically active radiation, 18–38 °C temperature range, 16–71% relative humidity range), and were watered daily. Harvest occurred 16 weeks after the beginning of the experiment.

2.4. Plant analysis

Entire plants were washed with tap water, followed by washing with HCl 0.1 M, and with de-mineralised water, separated in roots and shoots, after which root elongation and shoot length were registered. The biomass of the plants was determined after shoots and roots were oven dried at 70 °C for 48 h. Plants were then ground and sieved to <1 mm and shoot and root plant samples were digested at high temperatures (up to 330 °C) with a selenium and salicylic and sulphuric acids mixture for the determination of the levels of phosphorous and nitrogen in the plant tissues. Total nitrogen was determined by colorimetry, for which two reagents were added to 0.20 ml of the digests: 3 ml of reagent 1, consisting of a 5 M sodium nitroprusside solution and a 3 M antimonyl tartarate solution, and 5 ml of reagent 2, consisting of a mixture of a 1 M salicylate solution, a 1 × 10−3 M sodium nitroprusside solution and a 3 × 10−3 M EDTA solution. For total phosphorous colorimetric determination, two different reagents were added to 0.20 ml of the digest: 3 ml of reagent 1, consisting of a 3 × 10−3 M ascorbic acid solution and 1 ml of reagent 2 consisting of a mixture of a 6 × 10−3 M antimonyl tartarate solution, a 5 × 10−3 M ammonium molybdate solution, 0.7 M sulphuric acid and an anticoagulation agent (Wetting aerosol 22, Cytek, New Jersey, USA). The elements concentration on the resulting preparations was determined on a UNICAM HELIOS® spectrophotometer (Waltham, USA), at 660 nm for nitrogen and 880 nm for phosphorous (Wallenga et al., 1989).

2.5. Statistical analysis

Each test for the bacterial traits comprised 4 replicates. The greenhouse experiment was done with 5 bacterial treatments (control, 1ZP4, EC15, EC30, 1C2, ECP37^T and 3A10^T) and each treatment was replicated 4 times. Statistical analysis was performed using the SPSS program (SPSS Inc., Chicago, IL, Version 15.0). The data were analysed through analysis of variance (ANOVA). To detect the statistical significance of differences (P < 0.05) between means, the Duncan test was performed. Correlations were performed with different variables and Spearman's correlation coefficients were determined.

2.6. Chemicals

The chemicals used were analytical-grade and were obtained from Pronalab (Sintra, Portugal) — liquid reagents-, and Sigma–Aldrich (Missouri, USA) and Merck (Darmstadt, Germany) — solid reagents.

3. Results

3.1. Bacterial isolates traits

The tested phenotypic characteristics of strains 1ZP4, EC15, EC30, ECP37^T, 3A10^T and 1C2 are given in Table 2. The pH and temperature ranges for growth of the isolates were similar. Full length (about 1250–1450 bp) 16S rRNA of strains 1ZP4, EC15, EC30, ECP37^T, 3A10^T and 1C2 were sequenced and the closest affiliation according to sequencing is shown in Table 3. Strains 3A10^T and ECP37^T were already described (Pires et al., 2010) and correspond to the type strains of Chryseobacterium palustre and Chryseobacterium humi. Strains 1ZP4, EC15, EC30, and 1C2 were within the genera Sphingobacterium, Bacillus, Acromobacter, andRalstonia, respectively. Screening results of PGP traits of the selected bacteria are shown in Tables 4–6. IAA production was seen in all isolates, and at 48 h significant (P < 0.05) differences between the ability of the isolates to produce IAA could be translated by the expression 1C2 > 1ZP4 ≥ 3A10^T ≥ EC30 = EC15 = ECP37^T (Table 4); although the values remained comparable at 72 h, the differences of production shown at 48 h were faded, with only 1C2 showing significantly (P < 0.05) higher IAA production than the other isolates (Table 4). All the isolates showed positive results for HCN and NH3 production (Table 5); however, only isolates 1C2, 1ZP4 and ECP37^T had positive results for siderophore production. ACC-deaminase activity was detected only in some isolates, and (P < 0.05) differences between the ability of the isolates to degrade ACC into α-ketobutyrate could be translated by the expression ECP37^T > 3A10^T > 1C2 > EC30 = EC15 = 1ZP4 (Table 6).

3.2. Plant traits

Fig. 1 shows the effect of the application of different bacterial isolates in root and shoot elongation of Z. mays. The root elongation of the plants varied from a minimum of 40 (registered in the control group) to a maximum value of 55.6 cm (observed in the group of plants inoculated with EC30), with all the isolates, with the exception of 1C2, promoting significantly (P < 0.05) root growth when compared to control non-inoculated plants. Shoot elongation
ranged from a minimum length of 40.2 (observed in one of the replicates of the control plants) to a maximum of 59.0 bp (registered in a case of plants inoculated with 1ZP4), with all the isolates promoting significantly \((P < 0.05)\) shoot growth when compared to control plants; the isolates that better performed were 1ZP4, ECP37, \(^{1}\)C2, and \(^{2}\)C2.

The effect of the application of the six bacterial isolates on plant biomass production is shown in Fig. 2. The root dry biomass of \(Z\). \(m\)ays varied from 0.7289 (observed in one of the control plants) to 1.5028 g (registered in a case of plants inoculated with 1ZP4), with the isolates \(1\)C2 and \(2\)P4 promoting significantly \((P < 0.05)\) root biomass production when compared to control non-inoculated plants – the treatments with the remaining isolates did not show any significant effect \((P > 0.005)\) in comparison with control plants. For the shoot, the biomass ranged from 0.6214 (for a control plant) to 2.8424 g (for a plant replicate of the treatment with \(3\)A10), with all the isolates promoting significantly \((P < 0.05)\) shoot growth when compared to control plants; the isolates that better performed were \(3\)A10, \(^{1}\)C2, \(^{2}\)C2, and \(^{2}\)C2.

The levels of P in \(Z\). \(m\)ays roots and shoots are registered in Table 4. Phosphorous levels in the roots ranged from 404 (registered in the control plants) to 1417 mg liter\(^{-1}\) (observed in one of the treatments with the remaining isolates did not show any significant effect \((P < 0.001)\) in comparison with control plants. In the case of the shoot, the P levels increased \(P < 0.05\) accumulation was, as for root tissues, \(^{1}\)C2.

### Table 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>16S rRNA fragment length (bp)</th>
<th>Class of bacteria</th>
<th>Closest relatives</th>
<th>Similarity (%)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3)A10</td>
<td>1249</td>
<td>Flavobacteria</td>
<td>Chryseobacterium palustre</td>
<td>100</td>
<td>EU360967</td>
</tr>
<tr>
<td>ECP37(^{1})</td>
<td>1249</td>
<td>Flavobacteria</td>
<td>Chryseobacterium palustre</td>
<td>100</td>
<td>EU360967</td>
</tr>
<tr>
<td>(^{2})P4</td>
<td>1417</td>
<td>Sphingobacteria</td>
<td>Sphingobacterium sp. MG2</td>
<td>99</td>
<td>AVS56417</td>
</tr>
<tr>
<td>(^{2})C15</td>
<td>1405</td>
<td>Bacilli</td>
<td>Bacillus sp. K22-25</td>
<td>99</td>
<td>EU333888</td>
</tr>
<tr>
<td>(^{2})EC30</td>
<td>1350</td>
<td>(\beta) proteobacteria</td>
<td>Achromobacter sp., EP17</td>
<td>99</td>
<td>FJ027751</td>
</tr>
<tr>
<td>(^{1})C2</td>
<td>1450</td>
<td>(\beta) proteobacteria</td>
<td>Ralstonia eutropha H16</td>
<td>98</td>
<td>AM260479</td>
</tr>
</tbody>
</table>

### Table 5

<table>
<thead>
<tr>
<th>Isolate</th>
<th>HCN production</th>
<th>NH(_4) production</th>
<th>Siderophore production</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)C2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(3)A10</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>(3)EC30</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>(^{2})P4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(^{2})EC15</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>ECP37</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

ND – No color development detected; + – positive color development \((n = 4)\).

Fig. 3B represents the levels of N in \(Z\). \(m\)ays roots and shoots. Nitrogen levels ranged from 678 (registered in the control plants group) to 1750 mg kg\(^{-1}\) (observed in a replicate of the group of plants inoculated with \(1\)C2), with the isolates \(1\)C2, \(3\)EC30 and ECP37 promoting significantly \((P < 0.05)\) root N accumulation when compared to control non-inoculated plants. In the case of the shoot, the N levels ranged from 461 (in the control) to 1609 mg kg\(^{-1}\) (for a replicate in the \(^{2}\)C2 treated plants), with all the treatments promoting significantly \((P < 0.05)\) shoot accumulation of N when compared to control plants; the isolate that significantly \((P < 0.05)\) better performed was \(^{1}\)C2, as for P accumulation.

### 3.3. Interactions between plant parameters and bacterial traits

In the present study, IAA levels shown by the isolates at 72 h were generally positively related \((P < 0.05)\) with all plant traits (Table 7). The relation between siderophores, ammonia and HCN production and plant traits is also shown by the Spearman correlation coefficients presented in Table 7. The production of siderophores shown by the isolates was always positively related \((P < 0.05)\) with nitrogen accumulation and biomass of the plant tissues; ammonia and hydrogen cyanide production by the isolates was also positively and significantly \((P < 0.05)\) related to some plant characteristics such as elongation of roots (Spearman coefficients of 0.487 for both ammonia and HCN production) and shoots (Spearman coefficients of 0.595 for both bacterial traits) observed in the greenhouse experiment (Table 7).

### 4. Discussion

Plant rhizosphere is a preferential niche for various types of microorganisms in the soil. In the present investigation, 6 bacterial isolates were screened in vitro for their plant growth promoting (PGP) abilities. The isolates, named as 1ZP4, EC15, \(^{2}\)EC30, ECP37, 3A10 and \(^{1}\)C2 were identified as Sphingobacterium sp., Bacillus sp., Achromobacter sp., \(C\). \(h\)umi, \(C\). \(p\)alustre and Ralstonia eutropha. Rhizosphere species referred to promote corn growth include...
2007a) and species with growth promoting traits, e.g., *Sphingobacterium* in roots (Mehnaz et al., 2007c). Different studies have also reported

\[ P < 0.05 \] according to the Duncan test. The F-values of ANOVA for root and shoot lengths are $F_{6,21} = 2.297$ ($P < 0.05$) and $F_{6,21} = 9.287$ ($P < 0.001$), respectively.

*Pseudomonas putida* (Mehnaz and Lazarovits, 2006), *Glucanacetobacter azotocapitans* (Mehnaz and Lazarovits, 2006; Mehnaz et al., 2006) and several *Azospirillum* species — namely *Azospirillum zeae* (Mehnaz et al., 2007b), *Azospirillum canadense* (Mehnaz et al., 2007a) and *Azospirillum lipoforum* (Mehnaz and Lazarovits, 2006). *Sphingobacterium* species with growth promoting traits, e.g., *Sphingobacterium canadense*, have also been isolated from corn roots (Mehnaz et al., 2007c). Different studies have also reported strains from *Bacillus* species to be effective in promoting *Z. mays* growth, namely *Bacillus subtilis* (Araujo, 2008), and to protect other plants from diseases, such as wilt in tomato (Anith et al., 2004). Inoculation with *Chryseobacterium* species, e.g., *Chryseobacterium balusatrum*, has also been shown to enhance *Arabidopsis thaliana* plant growth and to protect against disease (Solano et al., 2008). The same was reported for the *Achromobacter* genus — a good example is the reduction of ethylene production and increased biomass in tomato plants inoculated with an isolate of *Achromobacter piechaudii* (Mayak et al., 2004a; Mayak et al., 2004b). In the case of *Ralstonia* species, some reports indicate some isolates as metal resistant (Goris et al., 2001), an ability with significant importance in plant growth promotion in disturbed environments, but the genus includes an important plant pathogen, *Ralstonia solanacearum* (Hayward, 1991).

Plants use phytohormones, such as auxins (e.g., indole acetic acid) to influence many cellular functions (Glick et al., 1999). All the isolates used in this study presented IAA production, most of the isolates generating levels comparable to those presented in other reports. Ahmad et al. (2008) reported levels of 2.13 and 3.6 mg l\(^{-1}\) for *Azotobacter* and *Pseudomonas* species, whereas Gravel et al. (2007) reported levels of 3.3 and 6.2 mg l\(^{-1}\) for *P. putida* and *Triochocerma atroviride*. However isolate 1C2, a *Ralstonia* species, showed much higher IAA production levels. IAA production by the isolates were positively related with all plant traits, with the exception of the correlation with root length that was not significant, probably due to the variable effect of IAA on root elongation. A low level of IAA produced by rhizobacteria promotes primary root elongation, whereas a high level increases lateral and adventitious root formation but inhibits the primary root growth (Xie et al., 1996). 1C2 treated plants appear as those with the highest root biomass production, which can be explained by the higher extent of adventitious roots. Plants inoculated with isolates with higher IAA production (namely 1C2), presented higher shoot elongation and also P and N accumulation in their tissues. Exogenous sources of IAA are responsible for changes in the morphology of the root system and influence the uptake of nutrients by the plant (San Francisco et al., 2005). This seemed to play an important role in

\[ F_6 = 12.093 (P < 0.001) \) and $F_{6,21} = 6.422 (P < 0.001)$, respectively.

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the present study, as P uptake by the plant increased when plants were treated with some of the isolates, especially 1C2 (Ralstonia sp.). Similar results have been reported by Gravel et al. (2007) for tomato plants inoculated with P. putida and T. atroviride.

1-Aminocyclopropane-1-carboxylate is an immediate precursor of ethylene in higher plants, and the production of the second is ultimately highly and positively dependent on endogenous levels of ACC (McKeon et al., 1982). Certain microorganisms can convert an enzyme ACC-deaminase that hydrolyses ACC into ammonia and α-ketobutyrate (Mayak et al., 1999) instead of its conversion into ethylene. Isolates 1C2, 3A10T and ECP37T presented the highest activity for ACC-deaminase. The uptake and cleavage of ACC by ACC-deaminase containing rhizobacteria decreases the amount of ACC, and consequently that of ethylene, therefore reducing the potentially inhibitory effects of higher ethylene concentrations (Glick et al., 1998), feature of extreme importance when plants are exposed to stressful conditions such as heavy metals contamination of the soil (Grichko et al., 2000). The activity of ACC-deaminase shown by our isolates was positively related with shoot and root biomass production and root length and N accumulation, seeming thus that the production of this enzyme can be influencing these plant traits. In fact, and according to the reports of authors such as Shaharroona et al. (2006), it seems that this influence is real for other plant and bacteria combinations, namely as the inoculation of pea seedlings with specific rhizobacteria containing ACC-deaminase had a positive effect concerning the increase of stem diameter and length and root elongation.

A number of plants possess heterologous iron uptake mechanisms (Yehuda et al., 1996). Masalha et al. (2000) found that plants cultivated under non-sterile conditions showed no iron-deficiency symptoms in contrast to plants grown in a sterile system, reinforcing the role of soil microbial activity in iron acquisition, namely through iron-bacterial siderophore complex generation. Other isolates from the Ralstonia, Sphingobacterium and Chryseobacterium species (1C2, 1ZP4 and ECP37T) showed siderophores production. Tian et al. (2009) also indicated Sphingobacterium species as siderophores producers. The promotion of plant growth is believed to occur by one or both of the following mechanisms: by directly supplying iron for plants – as the iron in the soil is present as insoluble ferric oxides, binding to siderophores produces soluble complexes (Glick et al., 1999) – or, as siderophores bind to the available form of iron in the soil, by rendering it unavailable to the plant pathogens (Ahmad et al., 2008). In the present study, the production of siderophores by the isolates seemed to influence the plant traits, as it was positively related with nitrogen accumulation, biomass and elongation of the shoots, and phosphorous and nitrogen accumulation and biomass of the roots.

It has been reported that overproduction of HCN may control fungal diseases in wheat seedlings (Flashman et al., 1996). All the isolates were positive for HCN production and although the greenhouse experiment was performed in sterile soil, this trait is very important when considering field applications, as plant resistance in a non-sterile environment will be potentially increased if the associated bacteria produce this component. The capacity of some bacterial species to produce ammonia also enhances plant growth. In the present study, all the isolates showed positive results for ammonia production. Ammonia and hydrogen cyanide production by the isolates was positively related to nitrogen accumulation and elongation of the roots, and phosphorous accumulation, biomass production and elongation of shoots. These bacterial traits can be influencing plant growth in numerous ways, although it is probably the combination of the PGP traits of the used species that is responsible by the increase in the assessed parameters in Z. mays. As the growth occurred in sterile soil, correlation coefficients found between HCN and plant traits in the greenhouse experiment may be explained by this interaction of plant growth promotion characteristics of the isolates as, generally, the tested isolates exhibited more than one PGP trait, which may promote plant growth directly or indirectly, or synergistically.

All the isolates used in the study presented at least one positive activity of plant growth promotion. Root elongation was increased in all inoculated plants, except for 1C2 (Ralstonia sp.), as discussed above. Shoot elongation and biomass production was promoted by all the isolates. However, when conjugating both plant traits, treatment with ECP37T and 1C2 isolates (C. humi and Ralstonia sp., respectively) outperformed the remaining ones. Results obtained in vitro for PGPR species may not be reproduced under field, semi-field or even greenhouse conditions. The performance of PGPR may be affected by climate and soil characteristics, amongst other factors. In the present study a positive effect of the 6 selected isolates on the growth and nutrient status of Z. mays plants in greenhouse assays was demonstrated when growth occurred in agricultural soil. The study occurred in an agricultural soil to ensure that the effects of the isolates in plant growth were not masked by possible effects of contaminants induced stress if a polluted matrix was used. However, future prospects of investigation include the application of Z. mays plants inoculated with the most promising strains — 1C2, 1ZP4 and ECP37T — in metal contaminated soil.

5. Conclusions

Inoculation with plant growth promoting bacterial isolates retrieved from a metal contaminated site enhanced the growth of Z. mays in greenhouse experiments, with R. eutropha, enhancing plant growth and nutrition by increasing shoot elongation and biomass by 24 and 100% respectively, and root biomass by 34%. Strains 1ZP4 and ECP37T from Sphingobacterium and Chryseobacterium species, have also shown good results — 1ZP4 increased shoot biomass and elongation by 57 and 31%, and root biomass and elongation by 46 and 16%, while ECP37T promoted shoot biomass production and elongation by 87 and 24%, and root biomass production and elongation by 49 and 21%. The biomass production of the plant shoots were correlated with IAA, HCN, ACC-deaminase activity and ammonia production of the isolates, considered as important plant growth promoting traits of rhizobacteria. Such isolates might have potential in future field applications as plant growth promoters.

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Table 7

<table>
<thead>
<tr>
<th>Plant traits</th>
<th>Bacterial traits</th>
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<tbody>
<tr>
<td>IAA (48 h)</td>
<td>IAA (72 h)</td>
</tr>
<tr>
<td>Shoot elongation</td>
<td>0.545* 0.622** 0.622**</td>
</tr>
<tr>
<td>Shoot biomass</td>
<td>0.581* 0.579* 0.518* 0.594* 0.594* 0.677**</td>
</tr>
<tr>
<td>Shoot P</td>
<td>0.751* 0.564<em>n.s. 0.505</em> 0.505** n.s.</td>
</tr>
<tr>
<td>Shoot N</td>
<td>n.s. 0.436* 0.625** n.s. n.s. n.s.</td>
</tr>
<tr>
<td>Root elongation</td>
<td>n.s. n.s. n.s. 0.487** 0.487** n.s.</td>
</tr>
<tr>
<td>Root biomass</td>
<td>0.383* 0.277* 0.777* n.s. n.s. 0.407**</td>
</tr>
<tr>
<td>Root P</td>
<td>0.499* 0.541* 0.616** n.s. n.s. n.s.</td>
</tr>
<tr>
<td>Root N</td>
<td>0.519* 0.676* 0.465* 0.606** 0.606** 0.406*</td>
</tr>
</tbody>
</table>

*Correlation is significant at the 0.05 level; n.s., no significant correlation.