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

Ana P.G.C. Marques[a], Carlos Pires[a,b], Helena Moreira[a], António O.S.S. Rangel[a], Paula M.L. Castro[a,*]

[a] Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal
[b] Cranfield Health, Cranfield University, Building 52A, Cranfield, Bedford MK430AL, England, UK

Keywords:
PGPR
Zea mays
HCN
Ammonia
IAA
Siderophores
Plant elongation
Plant biomass production
P and N accumulation

A B S T R A C T

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 $3A10^T$, $ECP37^T$, corresponding to *Chryseobacterium palustre* and *Chryseobacterium 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 $ECP37^T$ 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 $ECP37^T$ also led to increased plant growth in the greenhouse. These 3 species, corresponding to strains within the genera *Ralstonia* (1C2), *Sphingobacterium* (1ZP4), and to a strain identified as *C. humi* ($ECP37^T$) can thus be potential agents to increase crop yield in maize plants.

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-aminocyclopropane-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 N_2 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 goesingense*

* Corresponding author. Tel.: +351 22 558 00 59; fax: +351 22 509 03 51.
E-mail addresses: amarques@mail.esb.ucp.pt (A.P.G.C. Marques), cmpires@mail.esb.ucp.pt (C. Pires), helenamoreira@hotmail.com (H. Moreira), aorangel@esb.ucp.pt (A.O.S.S. Rangel), plcastro@esb.ucp.pt (P.M.L. Castro).

(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 phytoremediation 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.

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 $ECP37^T$ and $3A10^T$ (Pires et al., 2010) were used. Tests listed below were performed on all strains. The pH range for growth was determined in buffered trypticase soy broth (TSB) adjusted at pH 3–10 (at 1 pH unit intervals). The turbidity of the cultures grown in an orbital shaker at 25 °C was measured at 610 nm. All buffer solutions used to adjust the pH of TSB were prepared from 1 M stock solutions according to Gomori (1990). Citrate buffer was used for pH 3–6, phosphate buffer for pH 7, Tris buffer for pH 8, and a carbonate-bicarbonate buffer for pH 9 and 10. Growth temperature ranges were determined on TSA incubated at 4, 10, 15, 20, 25, 30, 37 and 50 °C. Extraction of genomic DNA, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described by Rainey et al. (1996). Cloning of the amplicons into pGEM T-Easy vector (Promega) and cycle-sequencing were performed at Macrogen Inc. (Seoul, Republic of Korea), using 16S universal bacterial primers (f27, f518, r800, r1492) (Lane, 1991). The quality of the 16S rRNA gene sequences was checked manually by the use of BioEdit program (version 7.0.5.3) (Hall, 1999), and the sequences were aligned against representative reference sequences of the most closely related members obtained from the National Center for Biotechnology Information database.

2.2. Assays 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 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 amending 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 Neilands, 1987).

ACC-deaminase activity was assayed according to a modification of the method of Honma and Shimomura (1978) which measures the amount of α-ketobutyrate produced upon the hydrolysis of ACC. The number of μmol of α-ketobutyrate produced by this reaction was determined by comparing to a standard curve of α-ketobutyrate ranging between 0.1 and 1 μmol 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® 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 μl of toluene were added to the cell suspension and vortexed for 30 s and 200 μl of the resulting suspension were placed in a fresh microcentrifuge tube, to which with 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) NaOCl 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

Table 1
Soil properties.

pH	6.44 ± 0.08
Water content (%)	4.84 ± 0.03
Organic content (%)	11.3 ± 0.2
N (mg kg^{-1})	4200 ± 150
P (mg kg^{-1})	260 ± 21
Mn (mg kg^{-1})	39 ± 2
K (mg kg^{-1})	10,600 ± 124
Zn (mg kg^{-1})	33 ± 2
Pb (mg kg^{-1})	8.7 ± 0.3
Cu (mg kg^{-1})	40 ± 2
Cr (mg kg^{-1})	40 ± 6
As (mg kg^{-1})	<5 (L.O.D.)
Cd (mg kg^{-1})	<5 (L.O.D.)
Hg (mg kg^{-1})	<0.01 (L.O.D.)
Ni (mg kg^{-1})	< 5 (L.O.D.)

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 grinded 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 mixture of a 5×10^{-2} M dissodiumhydrogenphosphate buffer (pH = 12.3) and a 4% bleach 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 1 ml of the digests: 3 ml of reagent 1, consisting of a 3×10^{-2} 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 (Wallinga et al., 1989).

2.5. Statistical analysis

Each test for the bacterial traits comprised 4 replicates. The greenhouse experiment was a design 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 (Darmstad, 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*, *Achromobacter*, and *Ralstonia*, 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 NH_3 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

Table 2
Characteristics of strains 3A10^T, ECP37^T, 1ZP4, EC15, EC30 and 1C2.

Characteristic	3A10^T	ECP37^T	1ZP4	EC15	EC30	1C2
Growth temperature (°C)						
Range	10–37	4–37	10–40	15–32	10–40	10–40
Optimum	30	25–30	25–30	25	30	25
pH for growth						
Range	6–9	6–9	5–9	6–8	5–9	5–9
Optimum	7	7–8	7	6–7	7–8	6–7

Table 3
Closest relatives (BLAST search) of strains 3A10^T, ECP37^T, 1ZP4, EC15, EC30 and 1C2.

Strain	16S rRNA fragment length (bp)	Class of bacteria	Closest relatives	Similarity (%)	Accession number
3A10^T	1249	*Flavobacteria*	*Chryseobacterium palustre*	100	EU360967
ECP37^T	1249	*Flavobacteria*	*Chryseobacterium humi*	100	EU360967
1ZP4	1417	*Sphingobacteria*	*Sphingobacterium* sp. MG2	99	AY556417
EC15	1465	*Bacilli*	*Bacillus* sp. K22-25	99	EU333888
EC30	1350	*β-proteobacteria*	*Achromobacter* sp., EP17	99	FJ827751
1C2	1450	*β-proteobacteria*	*Ralstonia eutropha* H16	98	AM260479

ranged from a minimum length of 40.2 (observed in one of the replicates of the control plants) to a maximum of 59 (registered for 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^T and 1C2.

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. mays* varied from 0.7289 (observed in one of the control plants) to 1.5008 g (registered in a case of plants inoculated with ECP37^T), with the isolates 1C2 and ECP37^T 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 3A10^T), with all the isolates promoting significantly ($P < 0.05$) shoot growth when compared to control plants; the isolates that better performed were 3A10^T, ECP37^T and 1C2.

The levels of P in *Z. mays* roots and shoots are registered in Fig. 3A. Phosphorous levels in the roots ranged from 404 (registered in the control treatment) to 1139 mg kg^{-1} (observed in for a plant inoculated with 1C2), with the isolates 1C2 and 1ZP4 promoting significantly ($P < 0.05$) root P accumulation when compared to control non-inoculated plants – the treatment with the 1C2 isolate significantly ($P < 0.05$) outperformed the remaining ones, followed by the treatment with 1ZP4. In the case of the shoot, the P levels ranged from 251 (in control) to 802 mg kg^{-1} (in the treatment with 1C2), with the isolates 1C2, EC15, 3A10^T and EC30 promoting significantly ($P < 0.05$) shoot accumulation of P when compared to control plants; the isolate that most significantly ($P < 0.05$) increased P accumulation was, as for root tissues, 1C2.

Table 5
In vitro screening results for cyanide, ammonia and siderophore production.

Isolate	HCN production	NH_3 production	Siderophore production
Control	ND	ND	ND
1C2	+	+	+
3A10	+	+	ND
EC30	+	+	ND
1ZP4	+	+	+
EC15	+	+	ND
ECP37	+	+	+

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

Fig. 3B represents the levels of N in *Z. mays* 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 1C2), with the isolates 1C2, EC30 and ECP37^T 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 1C2 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 1C2, 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, EC30, ECP37^T, 3A10^T and 1C2 were identified as *Sphingobacterium* sp., *Bacillus* sp., *Achromobacter* sp., *C. humi*, *C. palustre* and *Ralstonia eutropha*. Rhizosphere species referred to promote corn growth include

Table 4
Indole acetic levels (mg l^{-1}) of screened isolates at 48 and 72 h.

Isolate	IAA 48 h (mg l^{-1})	IAA 72 h (mg l^{-1})
1C2	14 ± 3^a	15 ± 3^a
3A10	5 ± 1^{bc}	5 ± 1^b
EC30	2,9 ± 0,6^c	4 ± 1^b
1ZP4	7 ± 4^b	6 ± 3^b
EC15	3,1 ± 0,5^c	5 ± 1^b
ECP37	3 ± 1^c	5,0 ± 0,3^b
	$F_{5,18} = 36{,}260$ ($P < 0.001$)	$F_{5,18} = 15{,}079$ ($P < 0.001$)

Results are expressed as means ± SD ($n = 4$). One way ANOVA was performed for each time. Means for the same time with different letters are significantly different from each other ($P < 0.05$) according to the Duncan test. IAA was never detected in control cultures which were thus not considered for this statistical analysis.

Table 6
ACC-deaminase activity (nmol α-ketobutyrate g^{-1} h^{-1}) of screened isolates.

Isolate	ACC-deaminase activity
1C2	6 ± 4^c
3A10	19 ± 9^b
EC30	n.d.
1ZP4	n.d.
EC15	n.d.
ECP37	57 ± 15^a
	$F_{5,18} = 36{,}260$ ($P < 0.001$)

Results are expressed as means ± SD ($n = 4$). Means with different letters are significantly different from each other ($P < 0.05$) according to the Duncan test. ACC-deaminase activity was never detected in control cultures which were thus not considered for this statistical analysis; n.d. means no activity was found.

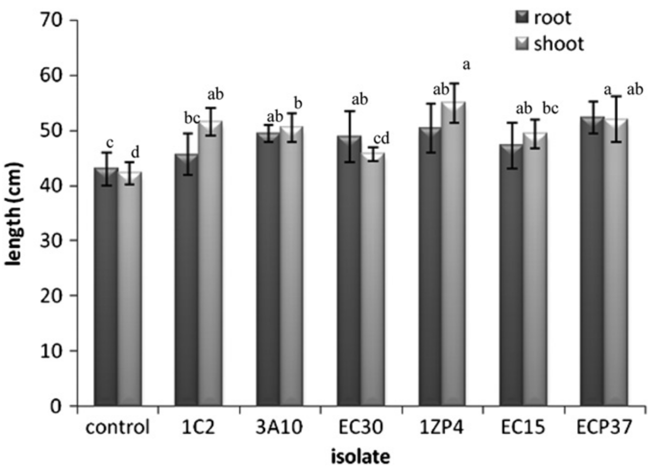

Fig. 1. Root and shoot elongation (cm) of *Zea mays.* Results are expressed as means ± SD ($n = 4$). One way ANOVA was performed for each plant section. Means for the same plant section with different letters are significantly different from each other ($P < 0.05$) according to the Duncan test. The *F*-values of ANOVA for root and shoot lengths are $F_{6,21} = 2.970$ ($P < 0.05$) and $F_{6,21} = 9.287$ ($P < 0.001$), respectively.

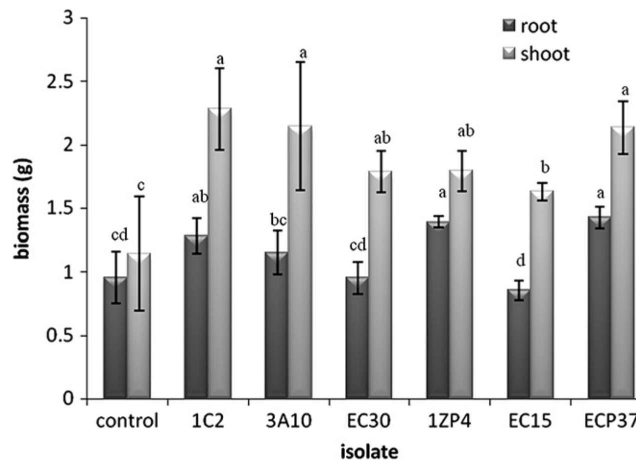

Fig. 2. Biomass of roots and shoots of *Zea mays*. Results are expressed as means ± SD ($n = 4$). One way ANOVA was performed for each plant section. Means for the same plant section with different letters are significantly different from each other ($P < 0.05$) according to the Duncan test. The *F*-values of ANOVA for root and shoot lengths are $F_{6,21} = 12.093$ ($P < 0.001$) and $F_{6,21} = 6.422$ ($P < 0.001$), respectively.

Pseudomonas putida (Mehnaz and Lazarovits, 2006), *Gluconacetobacter azotocaptans* (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 *Azospirilum lipoferum* (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 balustinum,* 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 *Trichoderma 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

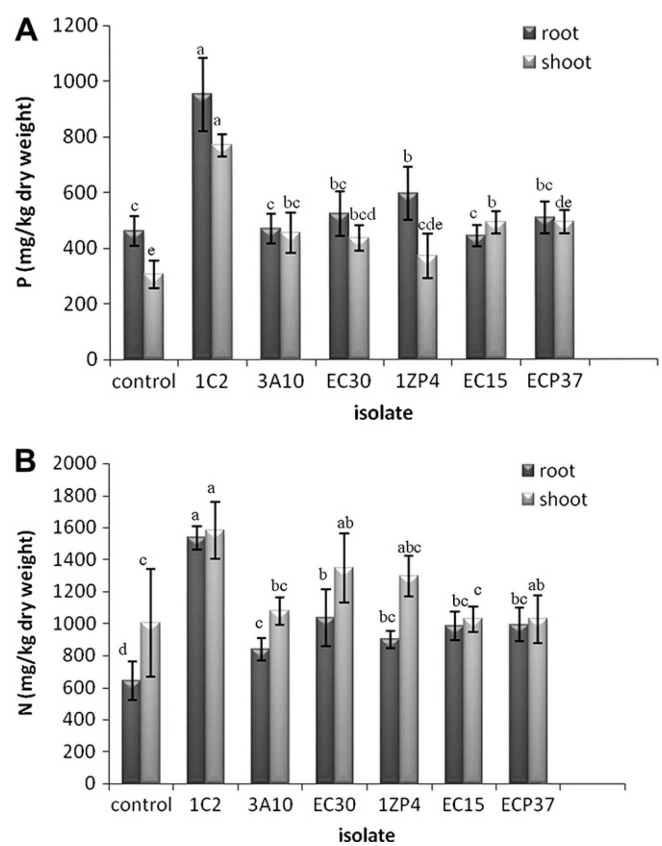

Fig. 3. P (A) and N (B) levels in *Zea mays* roots and shoots. Results are expressed as means ± SD ($n = 4$). One way ANOVA was performed for each plant section and for each element. Means for the same plant section with different letters are significantly different from each other ($P < 0.05$) according to the Duncan test. The *F*-values of ANOVA for root and shoot P contents (A) are $F_{6,21} = 20.705$ ($P < 0.001$) and $F_{6,21} = 30.852$ ($P < 0.001$), respectively. The *F*-values of ANOVA for root and shoot N contents (B) are $F_{6,21} = 26.517$ ($P < 0.001$) and $F_{6,21} = 5.193$ ($P < 0.005$), respectively.

Table 7
Spearman's correlation coefficients between plant and bacterial traits.

Plant traits	Bacterial traits					
	IAA (48 h)	IAA (72 h)	Siderophores	HCN	Ammonia	ACC-deaminase
Shoot elongation	0.517**	0.547**	0.622**	0.595*	0.595**	n.s.
Shoot biomass	0.581**	0.679**	0.518**	0.594**	0.594**	0.677**
Shoot P	0.751**	0.564**	n.s.	0.505**	0.505**	n.s.
Shoot N	n.s.	0.436*	0.625**	n.s.	n.s.	n.s.
Root elongation	n.s.	n.s.	n.s.	0.487**	0.487**	n.s.
Root biomass	0.383*	0.277*	0.777**	n.s.	n.s.	0.497**
Root P	0.499**	0.541**	0.616**	n.s.	n.s.	n.s.
Root N	0.519**	0.676**	0.465*	0.606**	0.606**	0.406*

**Correlation is significant at the 0.01 level; * correlation is significant at the 0.05 level; n.s., no significant correlation.

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 contain an enzyme ACC-deaminase that hydrolyses ACC into ammonia and α-ketobutyrate (Mayak et al., 1999) instead of its conversion into ethylene. Isolates 1C2, 3A10^T and ECP37^T 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 Shaharoona 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. Only isolates from the *Ralstonia*, *Sphingobacterium* and *Chryseobacterium* species (1C2, 1ZP4 and ECP37^T) 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 (Flaishman 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 increase 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 ECP37^T 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 ECP37^T – 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 ECP37^T 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 ECP37^T 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.

Acknowledgments

The authors wish to thank Câmara Municipal de Estarreja for the provision of access to the site. Ana Marques and Carlos Pires had the support of a Fundação para a Ciência e a Tecnologia grants SFRH/BPD/34585/2007 and SFRH/BD/25493/2005, respectively.

References

Abou-Shanab, R.I., Angle, J.S., Delorme, T.A., Chaney, R.L., van Berkum, P., Moawad, H., Ghanem, K., Ghozlan, H.A., 2003. Rhizobacterial effects on nickel extraction from soil and uptake by *Alyssum murale*. New Phytologist 158, 219–224.

Ahmad, F., Ahmad, I., Khan, M.S., 2008. Screening of free living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiological Research 163, 173–181.

Anith, K.N., Momol, M.T., Kloepper, J.W., Marois, J.J., Olson, S.M., Jones, J.B., 2004. Efficacy of plant growth promoting rhizobacteria, acibenzolar-s-methyk and soil amendment for integrated management of bacterial wilt on tomato. Plant Disease 88, 669–673.

Araujo, F.F., 2008. Seed inoculation with *Bacillus subtilis* formulated with oyster meal and growth of corn, soybean and cotton. Ciência Agrotecnologia Lavras 32, 456–462.

Cappuccino, J.C., Sherman, N., 1992. Negative staining. In: Cappuccino, J.C., Sherman, N. (Eds.), Microbiology: A Laboratory Manual, third ed. Benjamin/Cummings Pub Co, Redwood City, pp. 125–179.

Flaishman, M.A., Eyal, Z.A., Zilberstein, A., Voisard, C., Hass, D., 1996. Suppression of *Septoria tritci* blotch and leaf rust of wheat by recombinant cyanide producing strains of *Pseudomonas putida*. Molecular Plant and Microbe Interactions 9, 642–645.

Fuhrmann, J.J., Wollum, A.G., 1989. Nodulation competition among *Bradyrhizobium japonicum* strains as influenced by rhizosphere bacteria and iron availability. Biology and Fertility of Soils 7, 108–112.

Glick, B.R., 1995. The enhancement of plant growth by free living bacteria. Canadian Journal of Microbiology 41, 109–114.

Glick, B.R., Penrose, D.M., Li, J., 1998. A model for the lowering of plant ethylene concentrations by plant growth promoting bacteria. Journal of Theoretical Biology 190, 63–68.

Glick, B.R., Patten, C.L., Holquin, G., Penrose, D.M., 1999. Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria. Imperial College Press, London.

Gomori, G., 1990. Preparation of buffers. In: John, N.A., Malvin, I.S. (Eds.), Methods of Enzymology, vol. I. Academic Press Inc, San Diego, pp. 138–146.

Goris, J., De Vos, P., Coenye, T., Hoste, B., Janssens, D., Brim, H., Diels, L., Mergeay, M., Kersters, K., Vandamme, P., 2001. Classification of metal-resistant bacteria from industrial biotopes as *Ralstonia campinensis* sp. nov., *Ralstonia metallidurans* sp. nov. and *Ralstonia basilensis* Steinle et al. 1998 emend. International Journal of Systematic and Evolutionary Microbiology 51, 1773–1782.

Gravel, V., Antoun, H., Tweddell, R.J., 2007. Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonas putida* or *Trichoderma atroviride*: possible role of indole acetic acid (IAA). Soil Biology and Biochemistry 39, 1968–1977.

Grichko, V.P., Filby, B., Glick, B.R., 2000. Increased ability of transgenic plants expressing the bacterial enzyme ACC-deaminase to accumulate Cd, Co, Cu, Ni, Pb, and Zn. Journal Biotechnology 81, 45–53.

Grichko, V.P., Glick, B.R., 2001. Flooding tolerance of transgenic tomato plants expressing the bacterial enzyme ACC deaminase controlled by the 35S, rolD or PRB-1b promoter. Plant Physiology and Biochemistry 39, 19–25.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95–98.

Hayward, A.C., 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annual Reviews in Phytopathology 29, 65–87.

Honma, M., Shimomura, T., 1978. Metabolism of 1-aminocyclopropane-1-carboxylic acid. Agriculture Biology and Chemistry 42, 1825–1831.

Idris, R., Trifonova, R., Puschenreiter, M., Wenzel, W.W., Sessitsch, A., 2004. Bacterial communities associated with flowering plants on the Ni hyperaccumulator *Thlaspi goesingense*. Applied Environmental Microbiology 70, 2667–2677.

Kamnev, A.A., Lelie, D., 2000. Chemical and biological parameters as tools to evaluate and improve heavy metal phytoremediation. Bioscience Reports 20, 239–258.

Khan, A.G., 2005. Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. Journal of Trace Elements in Medicine and Biology 18, 355–364.

Lane, D.J., 1991. 16S/23S sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley, New Jersey, pp. 171–204.

Lin, Q., Shen, K.L., Zhao, H.M., Li, W.H., 2008. Growth response of *Zea mays* L. in pyrene-copper co-contaminated soil and the fate of pollutants. Journal of Hazardous Materials 150, 515–521.

Marques, A.P.G.C., Rangel, A.O.S.S., Castro, P.M.L., 2007. Zn accumulation in plant species indigenous to a Portuguese polluted site: relation with soil contamination. Journal of Environmental Quality 36, 646–653.

Masalha, J., Kosegarten, H., Elmaci, O., Mengel, K., 2000. The central role of microbial activity for iron acquisition in maize and sunflower. Biology and Fertility of Soils 30, 433–439.

Mayak, S., Tivosh, T., Glick, B.R., 1999. Effect of wild type and mutant plant growth promoting rhizobacteria on the rooting of mung bean cuttings. Journal Plant Growth Regulation 18, 49–53.

Mayak, S., Tirosh, T., Glick, B.R., 2004a. Plant growth-promoting bacteria that confer resistance in tomato plants to salt stress. Plant Physiology and Biochemistry 42, 565–572.

Mayak, S., Tirosh, T., Glick, B.R., 2004b. Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. Plant Science 166, 525–530.

McKeon, T.A., Hoffmann, N.E., Yang, S.F., 1982. The effect of plant-hormone pretreatments on ethylene production and synthesis of 1-aminocyclopropane-1-carboxylic acid in water-stressed wheat leaves. Planta 155, 437–443.

Meers, E., Van slycken, S., Adriaensen, K., Ruttens, A., Vangronsveld, J., Du Laing, G., Witters, N., Thewys, T., Tack, F.M.G., 2010. The use of bio-energy crops (*Zea mays*) for phytoattenunation of heavy metals on moderately contaminated soils: a field experiment. Chemosphere 78, 35–41.

Mehnaz, S., Lazarovits, G., 2006. Inoculation effects of *Pseudomonas putida, Gluconabacter azotocaptans* and *Azospirilum lipoferum* on corn plant growth under greenhouse conditions. Microbial Ecology 51, 326–335.

Mehnaz, S., Weselowski, B., Lazarovits, G., 2006. Isolation and identification of *Gluconabacter azotocaptans* from corn rhizosphere. Systematic and Applied Microbiology 29, 496–501.

Mehnaz, S., Weselowski, B., Lazarovits, G., 2007a. *Azospirillum canadense* sp. nov., a nitrogen fixing bacterium isolated from corn rhizosphere. International Journal of Systematic and Evolutionary Microbiology 57, 620–624.

Mehnaz, S., Weselowski, B., Lazarovits, G., 2007b. *Azospirillum zeae* sp. nov., a diazotrophic bacteria isolated from rhizosphere soil of *Zea mays*. Journal of Systematic and Evolutionary Microbiology 57, 2805–2809.

Mehnaz, S., Weselowski, B., Lazarovits, G., 2007c. *Sphingobacterium canadense* sp. nov., an isolate from corn roots. Systematic and Applied Microbiology 30, 519–524.

Pires, C., Carvalho, M.F., De Marco, P., Magan, N., Castro, P.M.L., 2010. *Chryseobacterium palustre* sp. nov. and *Chryseobacterium humi* sp. nov., isolated from industrially contaminated sediments. International Journal of Systematic Evolutionary Microbiology 60, 402–407.

Rainey, F.A., Ward-Rainey, N., Kroppenstedt, R.M., Stackebrandt, E., 1996. The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov. International Journal of Systematic Bacteriology 46, 1088–1092.

Reed, M.L.E., Glick, B.R., 2004. Applications of free living plant growth-promoting rhizobacteria. Antonie van Leeuwenhoek 86, 1–25.

Reed, M.L.E., Glick, B.R., 2005. Growth of canola (*Brassica napus*) in the presence of plant growth-promoting bacteria and either copper or polycyclic aromatic hydrocarbons. Canadian Journal of Microbiology 51, 1061–1069.

San Francisco, S., Houdusse, F., Zamarreno, A.M., Garnica, M., Casanova, E., Garcia-Mina, J.M., 2005. Effects of IAA and IAA precursors on the development, mineral nutrition, IAA content and free polyamine content of pepper plants cultivated in hydroponic conditions. Scientia Horticulturae 106, 38–52.

Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry 160, 47–56.

Shaharoona, B., Arshad, M., Zahir, Z.A., Khalid, A., 2006. Performance of *Pseudomonas* spp.containing ACC-deaminase for improving growth and yield of maize (*Zea mays* L.) in the presence of nitrogenous fertilizer. Soil Biology and Biochemistry 38, 2971–2975.

Solano, B.R., Maicas, J.B., Iglesia, M.T.P., Domenech, J., Manero, F.J.G., 2008. Systemic disease protection elicited by plant growth promoting rhizobacteria strains: relationship between metabolic responses, systemic disease protection and biotic elicitors. Phytopathology 98, 451–457.

Solomon, B.D., Barnes, J.R., Halvorsen, K.E., 2007. Grain and cellulosic ethanol: history, economics, and energy policy. Biomass and Bioenergy 31, 416–425.

Tiago, I., Teixeira, I., Silva, S., Chung, P., Veríssimo, A., Manaia, C., 2004. Metabolic and genetic diversity of mesophilic and thermophilic bacteria isolated from composted municipal sludge on poly-epsilon-caprolactones. Current Microbiology 49, 407–414.

Tian, F., Ding, Y., Zhu, H., Yao, L., Du, B., 2009. Genetic diversity of siderophore-producing bacteria of tobacco rhizosphere. Brazilian Journal of Microbiology 40, 276–284.

Vivas, A., Biró, B., Ruíz-Lozano, J.M., Barea, J.M., Azcón, R., 2006. Two bacterial strains isolated from a Zn-polluted soil enhance plant growth and mycorrhizal efficiency under Zn-toxicity. Chemosphere 62, 1523–1533.

Wallinga, I., Vark, W., Houba, V.J.G., Lee, J.J., 1989. Plant Analysis Procedures. Department of Soil Science and Plant Nutrition, Wageningen Agricultural University, Syllabus, Wageningen.

Whiting, S.N., de Souza, M.P., Terry, N., 2001. Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*. Environmental Science and Technology 15, 3144–3150.

Wohler, I., 1997. Auxin-indole derivatives in soils determined by a colorimetric method and by high performance liquid chromatography. Microbiological Research 152, 399–405.

Xie, H., Pastrenak, J.J., Glick, B.R., 1996. Isolation and characterization of mutants of the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2 that overproduce indoleacetic acid. Current Microbiology 32, 67–71.

Yehuda, Z., Shenker, M., Romheld, V., Marschner, H., Hadar, Y., Chen, Y., 1996. The role of ligand exchange in the uptake of iron from microbial siderophores by graminaceous plants. Plant Physiology 112, 1273–1280.