Ann Microbiol (2016) 66:1087–1097
DOI 10.1007/s13213-016-1194-6

ORIGINAL ARTICLE

Phylogenetic and plant-growth-promoting characteristics of *Bacillus* isolated from the wheat rhizosphere

Hafsa Cherif-Silini[1] · Allaoua Silini[1] · Bilal Yahiaoui[1] · Imen Ouzari[2] · Abdellatif Boudabous[2]

Received: 26 June 2015 / Accepted: 18 January 2016 / Published online: 2 February 2016

Abstract The rhizobacteria that promote the growth of plants can have a positive effect on the productivity of crops, especially in stress conditions. Among the plant -growth -promoting (PGP) rhizobacteria (PGPR) cluster, *Bacillus* spp. are among the genera with most potential due to their spore forming ability, thereby increasing the adaptation of *Bacillus* strains to commercial formulation and field application. Due to their intrinsic properties, the *Bacilli* have several mechanisms conferring beneficial effects on plants. Thirty-five strains of *Bacillus* isolated from the rhizosphere of wheat from three different soils in arid and semi-arid areas of Algeria were tested for properties involved in the promotion of plant growth. The PGP ability of the 35 strains was evaluated by determining their biofertilisation (phosphate solubilisation), biostimulation [indole acetic acid (IAA) production] and biocontrol [cyanhydric acid (HCN), siderophores, 2,3-butanediol production and antifungal activity] activities. Of the 35 strains, 78 % had the ability to solubilise phosphates at rates of 16.65 μg/mL for strain D13, 15.60 μg/mL for D7 and 15.05 μg/mL for D6. These strains were the most successful and were isolated from arid and alkaline soils. The highest concentrations of IAA were produced by strains D4 and D7 to values ranging from 10 to 19 μg/mL. All strains inhibited at least one fungal strain tested, and 75 % had activity against three fungi or more. More than half of *Bacillus* strains produced 2.3- butanediol but only a single strain produced HCN. Only three strains (B25, D11 and BA11) were efficient in the production of siderophores. Also, four strains (B21, D4, B10 and B25) possessed ACCdeaminase and were considered regulators of stress. Phylogenetic diversity of the strains was analysed by 16S rDNA sequencing. The results identified all strains as being similar to the *Bacillus* sp. cluster, and divided separately into five groups. The majority of strains ($n=28$) were assigned to the species *Bacillus thuringiensis* and *Bacillus subtilis*. The *Bacillus* species isolated in this study showing PGP abilities have the potential to be used as PGPR.

Keywords Bacilli · Plant growth promotion · 16S rRNA

✉ Hafsa Cherif-Silini
cherifhafsa@yahoo.fr

[1] Laboratoire de Microbiologie Appliquée, Département de Microbiologie, Faculté des Sciences de la Nature et de la Vie, Université Ferhat Abbas, Sétif-1, Algeria

[2] Laboratoire Microorganismes et Biomolécules Actives, Département de Biologie, Faculté des Sciences de Tunis, Campus Universitaire, 2092 Tunis, Tunisia

Introduction

The use of microorganisms in agriculture with the aim of improving nutrient availability for plants has become an important and necessary practice. Many species and specific strains of bacteria residing in the rhizosphere play an important role in plant growth promotion, increasing crop yields and reducing the incidence of disease. Bacteria associated with the roots of plants are defined as rhizobacteria. They are generally very able to colonise root systems, enriching available nutrients throughout the developmental cycle of the plant. These rhizobacteria are considered as effective microbial competitors in the root zone, and are often referred to as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al. 1989; Zahir et al. 2004).

Among the most commonly reported PGPR, strains of the genus *Bacillus* are prevalent (Qiao et al. 2014). In soil, the *Bacilli* represent a large fraction of the microbial community. They are found in the rhizosphere and as epiphytes or

endophytes in various crops, including wheat, corn, sorghum, sugarcane, barley, and forest trees (Holl et al. 1988). These rhizobacteria have the ability to produce many antibiotics, and are easy to grow in vitro or be manipulated in laboratory. In addition, the *Bacilli* have an advantage over other bacteria due to their ability to form endospores, and thus resist changes in environmental conditions leading to high stability as biofungicides or biofertilizers (Qiao et al. 2014).

PGPR used as fertilizers or biopesticides against plant pathogens are a promising alternative to chemical fertilisers and pesticides (Amar et al. 2013). However, the ability of bacteria to colonise roots and survive in the soil is often limited. As a consequence, the selection and use of PGPR must take into account the adaptation of the inoculant to a particular plant and ecosystem (Gamelero et al. 2010). The development of effective microbial inoculants remains a major scientific challenge.

Furthermore, the selection of effective PGPR strains is related to the characterisation of their properties promoting plant growth. These properties include: production of auxins and siderophores, nitrogen fixation, phytopathogenic antagonism, cyanogenesis, solubilisation of phosphate, and production of aminocyclopropane-1-carboxylate (ACC) deaminase activity (Ahemad and Kribet 2014). Therefore, isolation and screening of native strains is justified.

The aim of this study was to describe the plant growth promoting (PGP) activities of member of the genus *Bacillus* isolated from the wheat rhizosphere of different soils, and to determine their phylogenetic affiliation by 16S rDNA sequencing.

Materials and methods

Isolation of bacterial strains

Bacillus strains were collected from wheat rhizosphere in three areas in Algeria as follows: soil 1 was located in the North of Setif representing a semi-arid soil of the Ouricia region [36°17′40.5″N 25′32.8″E. pH 7.90, electrical conductivity (EC) = 1.3 mS/cm]; soil 2 was located in an arid area near a Sabkha in Boussaada region (35°23′38″N 4°19′18.1″E, pH 9 and EC = 3.57); and soil 3 was located in an arid zone in the region of Djelfa (35°01′28″N 3°50′10.2″E, pH 8.00 and EC = 3.01).

Soil adhering strongly to the roots of wheat was extracted, added to 10 mL sterile distilled water and shaken for 30 min. The processed soil sample was serially diluted, spread on nutrient agar (NA) plate at 30 °C/48 h. The colonies were subjected to repeated culturing several times to obtain pure cultures. Strains of the genus *Bacillus* were verified by the Gram stain and the presence of endospores. Pure cultures were kept at 4 °C on NA slants. A total of 35 strains was collected and distributed as follows: 17 strains (B) from soil 1, 9 strains (BA) from soil 2, and 9 strains (D) from soil 3.

In vitro screening for PGP activities

NH_3 and cyanhydric acid production

The ammonia production was revealed by addition of Nessler's reagent giving a yellow-to-brown color of peptone water inoculated by bacterial cultures and incubated at 30 °C for 48–72 h (Cappuccino and Sherman 1992).

Cyanhydric acid (HCN) production was indicated by development of an orange-brown colour on Whatman paper soaked in a solution of sodium picrate (5 % picric acid and 2 % sodium carbonate) placed on the top of the plate. This colour resulted from the reaction between the picrate sodium reagent and the hydrogen cyanide released by the bacterial growth cultures on NA amended with 4.4 g glycine/L and incubated at 30 °C for 2 days (Lorck 1948).

Nitrogen fixation

Molecular nitrogen fixation was tested on nitrogen-free medium. The DF salt minimal medium (Dworkin and Foster 1958) was inoculated by streaking from bacterial cultures, and incubating at 30 °C for 48 h. Any growth on the medium reflected the ability of bacteria to fix nitrogen.

Acetoin production

The production of acetoin was revealed by the Voges Proskauer (VP) reaction on Clark and Lubs medium. The medium was inoculated with 100 μL bacterial culture. After incubation at 30 °C for 24 h, the reagents VPI and VPII were added, and red color indicated presence of acetoin.

Phosphate solubilisation

The ability of the strains to solubilise phosphates was tested on Pikovskaya (PVK) medium containing tricalcium phosphate (Ca_3HPO_4) as the sole source of phosphate. A volume of 10 μL bacterial culture was deposited as a spot on the surface of the PVK agar as described by Gaur (1990). After incubation at 30 °C for 7 days, the diameter of the halo around the colony was measured. Quantitative analysis of tricalcium phosphate solubilisation in liquid medium was carried out on PVK liquid inoculated with 100 μL of each culture and incubated at 30 °C for 4 days. The cultures were then centrifuged at 5000 rpm for 15 min. The amounts of soluble phosphate were measured by the colorimetric method of Olsen (Olsen and Sommers 1982). The concentration of phosphate was determined by the absorbance of the blue colour at

610 nm. A standard calibration curve was prepared with a solution of KH_2PO_4.

Indole acetic acid production

The production of indole acetic acid (IAA) was tested on DF medium supplemented with 1 g/L tryptophan. The medium was inoculated with 100 μL bacterial culture and incubated at 30 °C for 72 h. A colorimetric assay was performed using the method of Loper and Scroth (1986). The cultures were centrifuged at 5000 rpm for 20 min; 1 mL supernatant was mixed with 2 mL Salkowski reagent (50 mL perchloric acid and 1 mL 35 % $FeCl_3$ 0.5 M). The OD was measured at 530 nm. Concentrations of IAA were determined using a calibration curve prepared from a IAA solution in the range 0 to 10^{-5}M.

Siderophore production

The production of siderophores was tested in Chrome Azurol S (CAS) medium (Shwyn and Neilands 1987). The King B medium, given its composition free of iron, was used to demonstrate the production of siderophores. The King B solid medium was inoculated with 10 μL bacterial culture and incubated at 30 °C for 24 h. After growth, 15 mL CAS agar was poured onto the bacterial culture. After a few hours, a change of colour from blue to orange appeared around any colony producing siderophores. The diameter of the orange halo was determined by subtracting the diameter of the colony from the total diameter (halo + colony). Quantitative analysis was performed on King B liquid medium inoculated with 100 μL culture and incubated at 30 °C for 72 h. Cultures were centrifuged at 5000 rpm for 30 min, and 500 μL of the supernatant was mixed with 500 μL CAS solution. The colour changed from blue to orange at the rate of siderophore production. The OD was measured by a spectrophotometer at 630 nm after 20 min of incubation.

The percentage of siderophores was calculated using the following formula:

St-Se/St x 100, where St is the CAS solution of colour intense blue (control), and Se is solution of the sample of less blue to orange depending on the intensity of production.

Antifungal activity

Antagonism activity was tested in vitro on potato dextrose agar (PDA) medium. Bacterial strains were tested for their ability to inhibit the growth of phytopathogenic fungi: *Aspergillus niger* (AN), *Fusarium oxysporum* fsp. Albedinis (FOA), *Alternaria alternata* (ALT), *Phytophthora infestans* (PI), *Botrytis cinerea* (B), and *Fusarium solani* (FS). An agar disc 6 mm in diameter from fresh culture of each fungus was deposited at the centre of the plate and 2 μL of each bacterial culture (10^8UFC/mL) was placed equidistantly at 3 cm from the fungal strain spot. Control plates that were not inoculated with bacteria were also prepared. Plates were incubated at 25 °C for 4 days. Fungal growth inhibition was quantified by using the percentage inhibition formula : $[(R-r) \times R^{-1} \times 100]$, where, r is the radius of the fungal colony that grew towards the bacterial colony, and R is the maximum growth that the fungus had in the Petri dish (Kumar et al. 2002).

Production of ACC-deaminase

ACC deaminase activity was determined by the ability of bacterial strains to use ACC as sole source of nitrogen on minimal salt medium (MSM) (Glick et al. 1994). According to the method described by Jacobson et al. (1994), the bacterial strains cultured in the presence of ACC and magnesium sulfate ($MgSO_4$) mineral source were compared according to their rate of growth. ACC deaminase activity was positive for strains having an OD greater than that of the $MgSO_4$ solution, which indicated the use of ACC as a source of nitrogen.

Genotypic characterization and identification

DNA extraction and PCR conditions

Total genomic DNA was extracted by a modified CTAB–SDS lysis protocol (Wilson 1990). PCR amplification of the 16S rRNA gene was performed using the universal primers 27 F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1496R (5′-CTACGGCTACCTTGTTACGA-3′) (Edwards et al. 1989). The PCR mixture (25 μL) contained 1x *Taq* polymerase buffer, 2.5 mmol/L $MgCl_2$, 0.1 mmol/L of each dNTP, 0.5 mmol/L of each primer, 1.25 U *Taq* DNA polymerase, and 10–100 ng genomic DNA.

The amplification was carried out in a thermocycler (Gene Amp PCR System 9700, Applied Biosystems, Tunis, Tunisia). The cycling conditions were as follows : 94 °C for 5 min followed by 35 cycles at 94 °C for 45 s, 55 °C for 1 min, 72 °C for 1 min, and with a final extension step at 72 °C for 10 min (Young et al. 1991). PCR products were separated in standard 1.5 % agarose gel in 0.5× tris-borate-EDTA buffer containing 1 μg/mL ethidium bromide.

16S rRNA sequencing and phylogenetic analysis

PCR-amplified 16S rDNA was purified by enzymatic treatment and sequenced by an automated DNA sequencer (ABI Prism 3130, Applied Biosystems). Partial 16S rRNA sequences of the strain isolates were compared with available sequences by the BLAST search (Saitou and Nei 1987) in the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were performed using ClustalW version 1.8 (Thompson et al. 1994). A phylogenetic dendrogram was

constructed using the neighbour-joining method, and tree topology was evaluated by performing bootstrap analysis of 1000 data sets using MEGA6.06 (Molecular Evolutionary Genetics Analysis) (Tamura et al. 2011).

Statistical analysis

Statistical analysis on the effects the bacterial strain on PGP traits (IAA and siderophores production, phosphate solubilisation and antifungal activity) was performed. The data were treated by analysis of variance (ANOVA), and means were compared by Tukey test. Principal component analysis (PCA) was performed by using Past version 2.07 software to determine correlations between different PGP activities.

Results

Plant growth promoting traits of isolates

Screening results of PGP traits of different *Bacillus* strains are shown in Figs. 1 and 2. NH_3 production occurred in the majority of strains (85 %) at greater rates, as assessed by the change in the intensity of the color to yellow from orange, after addition of Nessler reagent. The ability to solubilise phosphates was detected in 77 % of the isolates, and 60 % of the strains tested produced ≥ 1 μg/mL IAA. Nitrogen fixation capacity was evaluated by growth on nitrogen-free medium. This capacity was found in approximately half (48 %) the strains (Fig. 1). Indirect PGP activities were represented mainly by the antifungal activity results, where 77 % of the *Bacillus* strains showed inhibition against three or more fungi, and 57 % of the strains had the ability to produce siderophore units (%) ≥ 10 % on CAS medium. As regards volatile compounds ,including 2,3-butanediol and HCN, 57 % of the strains produced 2,3-butanediol, whereas only one strain (D13) produced HCN (2.85 %) (Fig. 2).

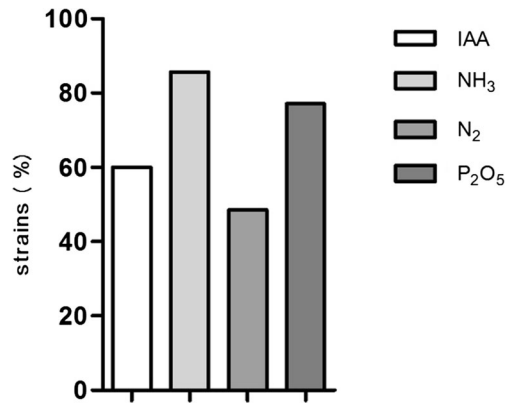

Fig. 1 Direct plant -growth-promoting (PGP) activities of test isolates

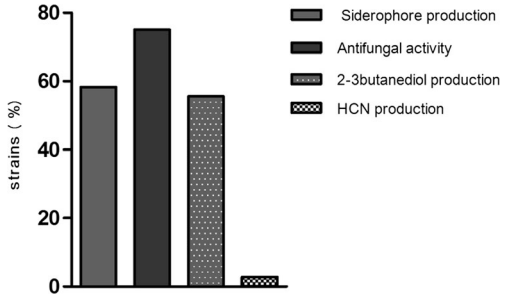

Fig. 2 Indirect PGP activities of test isolates

Phosphate solubilisation

Analysis of variance showed that the effect of strains on the solubilisation of phosphate was very significant ($P < 0.01$) (Table 1). The amount of P_2O_5 varied from 0 to 16.65 μg/mL. The maximum concentration of P_2O_5 soluble was observed in D13 strain (16.65 μg/mL) followed by D7 (15.60 μg/mL) and D6 (15.05 (μg/mL) strains (Table 2). On solid medium, the capacity for solubilisation was revealed by a transparent halo around the colony. Only three strains were given a halo of solubilisation, whereby the diameter formed by D6 was the largest (8 mm) followed by D13 (5 mm).

IAA production

Analysis of variance showed that the effect of strain on the production of IAA was significant at $P < 0.01$ (Table 1). According to the data in Table 2, *Bacillus* strains had very diverse production rates. These variations ranged from 0 μg/mL (B6, B15, B16, B19) to 19.50 μg/mL. Strains with maximum production were D4 (19.5 μg/mL) and D7(10.35 μg/mL).

Siderophore production

Quantitative estimation of siderophore production of the strains varied significantly ($P < 0.01$) (Table 1). Indeed, the percentage of siderophore production ranged from 1.28 to 53.05 %. The most powerful strains were B25 (53.05 %), BA11(51.51 %) and D11(43.5 %) (Table 2). An orange halo on solid medium, indicating siderophore production, was seen exclusively with strains BA11, D12 and D13.

Table 1 Analysis of variance summaries (mean squares) of data for indole acetic acid (IAA) production, siderophore production and phosphate solubilisation. *df* Degree of freedom

Source of variation	df	IAA	SID	P_2O_5
Strains	35	77.03622**	467.99217**	68.87486**
Error	36	1.73145	0.02651	0.56407

**Significant a level of 1 % ($P < 0.01$)

Table 2 Analysis of variance of multiple plant-growth-promoting (PGP) attributes of the *Bacillus* strains. Values with the same lower case letter do not differ significantly according to the Tukey test ($P > 0.05$). Values in parenthesis indicated the size of diameter (mm) on respective solid media. *SID* Siderophores, *IAA* indole acetic acid, *AFA* antifungal activity

Strains	SID (%)	P_2O_5(μg/mL)	IAA (μg/mL)	AFA ($n = 6$)
B1	17.06 h	9.95 f	1.15 ef	4 ab
B2	5.05 m	8.30 g	1.00 ef	5 ab
B4	9.81 l	5.34 h	0.90 ef	4 ab
B5	5.84 m	ND*	0.20 g	2 bc
B6	4.08 m	ND	ND	4 ab
B7	13.72 I	2.89 I	9.65 c	3 bc
B8	5.33 m	5.96 h	0.85 ef	3 bc
B10	19.46 h	5.87 h	0.65 fg	4 ab
B14	6.16 m	15.09 ab	2.30 de	2 bc
B15	0.47 n	8.60 g	ND	4 ab
B16	25.25 g	13.99 bc	ND	2 bc
B17	10.72 l	ND	0.20 g	**6** a**
B19	19.43 h	ND	ND	4 ab
B21	24.96 g	4.40 h	0.40 fg	5 ab
B22	12.00 j	ND	0.75 fg	4 ab
B23	25.23 g	5.39 h	0.43 fg	5 ab
B25	**53.05** b	ND	1.40 ef	**6** a
BA3	18.03 h	ND	2.20 de	4 ab
BA7	36.81 e	14.42 bc	2.30 de	**6** a
BA8	5.71 m	8.32 g	2.60 de	3 bc
BA9	10.58 l	ND	3.50 de	4 ab
BA11	**51.51** c (20)	13.55 cd	3.95 d	**6** a
BA12	25.07 g	13.99 cd	2.20 de	2 bc
BA13	1.28 n	13.52 cd	3.45 de	2 bc
BA14	6.59 m	13.66 cd	2.75 de	2 bc
BA15	1.69 n	12.86 de	2.20 de	5 ab
D1	7.10 m	13.84 cd	1.35 ef	4 ab
D4	6.01 m	13.67 cd	**19.50** b	1 c
D5	2.38 n	13.69 cd	2.50 de	4 ab
D6	32.96 f	**15.05** ab(8)	0.70 ef	5 ab
D7	15.95 I	**15.60** ab	**10.35** c	4 ab
D11	**43.50** d	13.80 cd	1.25 ef	5 ab
D12	10.46 l (2)	12.30 e	0.60 fg	4 ab
D13	13.97 I (2)	**16.65** a(5)	3.60 de	**6** a
D14	1.45 n	13.68 cd	1.55 ef	1 c

*Not detected

**Bold values indicate highest inhibition rates

Antifungal activity

The antifungal activity of *Bacillus* strains was tested on PDA medium against six fungi known for their phytopathogenic character: *Aspergillus niger* (AN), *Fusarium oxysporum* fsp. Albedinis (FOA), *Alternaria alternata* (ALT), *Phytophthora infestans* (PI), *Botrytis cinerea* (B), and *Fusarium solani* (FS). All *Bacillus* strains had activity against at least one fungal strain or more. The percentage of inhibition varied from 10.71 to 74.28 %. The fungi most sensitive to the *Bacillus* strains were PI, FS and ALT (Fig. 3). Five strains (BA7, B17, BA11, D13 and B25) had activity on all the fungal strains, while 15 strains inhibited ALT at a rate higher than 50 %. The most successful strains were D13 and D11, with 70 % inhibition. Antagonism to PI was induced by D1 and B1 strains at 60 %. The most active strains against AN were B8, B10, B21, and B23. The B17, D13 and BA11 strains had 54 % inhibition to *B. cinerea*. However, lower rates of inhibition were noted to FOA and FS, which were inhibited only by D1 and D11, and only at about 50 % (Table 3).

AAC-deaminase production

The production of ACC deaminase showed interesting features from the point of view of biocontrol activity, phytostimulation (IAA production), and biofertilisation (phosphate solubilisation). The results showed that only four strains D4, B10, B21 and B25 were able to use ACC as the sole nitrogen source (Table 4).

Principal component analysis

Principal component analysis (PCA) based on the obtained matrix was used to compare the amounts of PGP activities expressed by the strains. The results obtained explained variability of 42.76 % and 28.70 % according to the first and second PC, respectively (Fig. 4). Analysis of the data allowed the demonstration of a positive correlation between the production of IAA and phosphate solubilisation, and between antifungal activity and the production of siderophores. Concerning the behaviour of strains and their activities, this analysis allowed several groups to be distinguished.

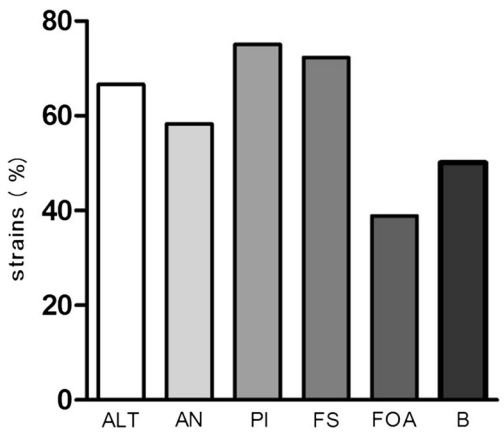

Fig. 3 Activity (%) of *Bacillus* strains against different fungal strains

Table 3 Growth inhibition (%) of the antagonistic *Bacillus* strains

Strains	B(%)	ALT(%)	AN(%)	PI(%)	FS(%)	FOA(%)
B1	38.09	ND	ND	**57.50**	32.14	44.11
B2	ND	ND	ND	ND	14.28	ND
B4	47.61	48.57	**57.14**	25.00	ND	ND
B5	ND	**68.57**[a]	ND	12.50	ND	ND
B6	23.80	ND	33.33	37.50	21.42	ND
B7	ND	**57.14**	ND	20.00	10.71	ND
B8	ND	ND	**64.28**	32.50	21.42	ND
B10	ND	ND	**59.52**	35.05	35.71	29.41
B14	ND	25.71	ND	ND	14.28	ND
B15	40.47	37.14	ND	40.00	17.85	ND
B16	**52.38**	ND	ND	30.02	ND	ND
B17	**54.76**	**51.42**	**57.14**	**55.01**	42.85	44.11
B19	ND	**62.85**	**52.38**	**52.50**	42.85	**50.00**
B21	ND	**57.14**	**59.52**	37.5	21.42	11.76
B22	28.57	20.00	40.47	ND	10.71	ND
B23	ND	28.57	**59.52**	12.50	17.85	12.94
B25	45.23	**51.42**	**52.38**	37.50	39.28	26.47
BA3	47.61	ND	40.47	37.50	35.71	ND
BA7	**50.00**	**62.85**	40.47	37.50	32.14	32.35
BA8	ND	28.57	28.57	ND	17.85	ND
BA9	ND	**65.57**	ND	**50.00**	46.42	44.11
BA11	**57.14**	**65.57**	**52.38**	37.50	28.57	35.29
BA12	23.80	ND	11.90	ND	ND	ND
BA13	ND	ND	28.57	37.50	ND	ND
BA14	ND	ND	45.23	ND	10.71	ND
BA15	35.71	37.14	38.09	37.50	ND	11.76
D1	ND	**57.14**	ND	**62.50**	46.42	**50.00**
D4	ND	28.57	ND	ND	ND	ND
D5	ND	42.85	ND	32.50	17.85	26.47
D6	47.61	**65.71**	ND	**50.04**	25.00	17.85
D7	**52.38**	11.42	**52.38**	42.50	ND	ND
D11	47.61	**74.28**	ND	47.50	**50.00**	**55.88**
D12	19.52	**54.28**	47.61	ND	17.85	ND
D13	**54.95**	**71.42**	**57.14**	**50.01**	39.28	47.05
D14	ND	57.14	ND	ND	ND	ND

[a] Bold values indicate highest inhibition rates

[b] Not detected

According to PC1 and PC2, three major groups were shown. Group 1 is represented by strains D4 and D7, which simultaneously produced IAA and phosphate solubilising activity. Strains BA11, D11, BA7, D6 and B25 were group 2, with a broad spectrum of antifungal activity and producing significant amounts of siderophores. The third group was represented by one strain, D13, which seemed to possess all activities. As for the other strains, they had a variable profile, which was mainly antifungal.

Phylogenetic analysis

A phylogenetic analysis was performed by partial 16S rRNA gene sequencing. Following BLAST analysis to search for homology, the sequences together with their closest relatives in GenBank were used to construct a phylogenetic tree using the neighbour-joining method (Fig. 5). Five phylogenetic groups related to distinct species or genera in the *Bacillaceae* family could be distinguished. The first group was related to *B. thuringiensis* and contained 19 isolates provided from the three soils. The second group including 12 isolates was that of *B. subtilis*. The other three groups were represented by one or two isolates each. BA9 was 99 % similar to *Brevibacterium frigoritolerans*. Strains B1 and B2 were related to *Paenibacillus* genus and D5 was identified as *Brevibacillus laterosporus* (Table 4).

Discussion

The *Bacilli* represent a significant fraction of the microbial community in soil. Sporulation of this bacterial genus promotes their ubiquity and survival in many various environments. Several studies on the diversity of the *Bacilli* in the rhizosphere of cereals have revealed that rhizosphere competence is a characteristic of each individual strain, not only of species or genus. Such studies have found that *Bacillus* species predominate in the rhizosphere of wheat and barley (Maplestone and Campbell 1989). Besides their ability to sporulate, *Bacillus* strains have several characteristics that improve their survival in the rhizosphere and thus their effectiveness as PGPR. This gives them a real advantage in the rhizosphere and provides diversity to their modes of action. They can display almost all the mechanisms of biological control, biostimulation and biofertilisation. In the present investigation, 35 strains of *Bacillus* sp. isolated from the rhizosphere of wheat were screened in vitro for PGP activities.

Nitrogen fixation is an important feature that can influence plant growth directly. However, 48 % of isolated *Bacillus* were capable of fixing atmospheric nitrogen. This activity is related to the existence of the *nif* H gene. This gene has been shown to exist in all species of *Bacillus* and *Peanibacillus* (Ding et al. 2005). In contrast, the production of NH_3 was more common in isolated strains; 85 % exhibited strong production of ammonia, which is taken up by plants as a source of nitrogen for their growth. This rate was comparable to that found in several works (e.g. Joseph et al. 2007).

Indole-3-acetic acid is the most common and most effective plant growth hormone. In addition to plants, about 80 % of rhizosphere bacteria are capable of producing IAA. Strains that produce a large amount of IAA, and acetamide indole (IAM), in soil increase the growth and yield of crops. It has been reported that plants, under certain constraints, are

Table 4 PGP attributes of *Bacillus* strains isolated from wheat rhizosphere

Isolate code	SID	P_2O_5	IAA	ACC	Antagonism assay[a]			Homology with	% identity	Accession number
					ALT	FS	PI			
B1	+	+	+	-	-	++	+++	*B. thuringiensis*	99	KM892531
B2	+	+	+	-	-	+	-	*Paenibacillus amylolyticus*	99	KM892532
B4	+	+	+	-	+++	-	+	*B. thuringiensis*	99	KM892533
B5	+	-	+	-	+++	-	+	*B. subtilis*	100	KM892534
B6	+	-	-	-	-	+	++	*B. thuringiensis*	99	KM892535
B7	+	+	+	-	+++	+	+	*B. thuringiensis*	99	KM892536
B8	+	+	+	-	-	+	++	*B. thuringiensis*	99	KM892537
B10	+	+	+	+	-	++	++	*B. thuringiensis*	97	JX196365
B14	+	+	+	-	+	+	-	*B. pumilus*	100	KM892538
B15	+	+	-	-	++	+	++	*B. pumilus*	99	KM892539
B16	+	+	-	-	–	-	-	*B. thuringiensis*	99	KM892540
B17	+	-	+	-	+++	+++	+++	*B. licheniformis*	99	KM892541
B19	+	-	-	-	+++	+++	+++	*B. subtilis*	99	KM892542
B21	+	+	+	+	+++	+	++	*B. thuringiensis*	97	JX196350
B22	+	+	+	-	+	+	-	*B. pumilus*	99	KM892543
B23	+	+	+	-	+	+	+	*B. thuringiensis*	99	KM892544
B25	+	-	+	+	+++	++	++	*B. thuringiensis*	97	JX196352
BA3	+	-	+	-	-	++	++	*B. thuringiensis*	97	JX196355
BA7	+	+	+	-	+++	++	+++	*B. subtilis* subsp. *subtilis*	99	JX196364
BA8	+	+	+	-	+	+	+	*B. thuringiensis*	100	KM892545
BA9	+	-	+	-	+++	+++	+++	*Brevibacterium*	99	JX196358
BA11	+	+	+	-	+++	+	+++	*B. thuringiensis*	97	JX196360
BA12	+	+	+	-	-	-	-	*B. subtilis* subsp *spizizenii*	99	JX196356
BA13	+	+	+	-	-	-	++	*B. anthracis*	99	KM892546
BA14	+	+	+	-	-	+	-	*B. thuringiensis*	99	KM892547
BA15	+	+	+	-	++	-	++	*B. thuringiensis*	99	KM892548
D1	+	+	+	-	+++	+++	+++	*B. thuringiensis*	99	KM892549
D4	+	+	+	+	+	-	-	*B. thuringiensis*	97	JX196351
D5	+	+	+	-	+++	+	++	*Brevibacillus laterosporus*	99	KM892550
D6	+	+	+	-	+++	-	+++	*B. subtilis* subsp. *subtilis*	99	JX196354
D7	+	+	+	-	+	-	+++	*B. subtilis* subsp. *subtilis*	99	JX196361
D11	+	+	+	-	+++	+++	+++	*B. subtilis* subsp. *subtilis*	99	JX196363
D12	+	+	+	-	+++	+	-	*B. thuringiensis*	97	JX196357
D13	+	+	+	-	+++	++	+++	*B. atropheaus*	99	GQ478020
D14	+	+	+	-	+++	-	-	*B. thuringiensis*	97	JX196359

[a] + low inhibition percentage (<30 %), ++ moderate inhibition percentage (30 % ≤ x ≤ 40 %), +++ strong inhibition percentage (>40 %), - no inhibition activity

dependent mainly on exogenous sources of phytohormones including those synthesized by bacteria. Strains D4 and D7 produced relatively high levels of IAA compared to all the other strains. These rates were comparable to those reported by several authors (Ertürk et al. 2010). The addition of tryptophan to the culture medium is necessary for the increased synthesis of this auxin. L-Tryptophan is considered as the precursor. Root exudates are a natural source of L-tryptophan for the microflora of the rhizosphere. The production of this compound can be variable among strains of different species. This variation is also influenced by the culture conditions, growth phase and substrate availability. According to Barazani and Friedmann (1999), bacteria capable of secreting a rate higher than 13.5 μg/mL indole compounds, are considered as PGPR. However, small amounts of IAA (10^{-9} to 10^{-12} M) are required for primary root growth (Vacheron et al. 2013). Indeed, the IAA rate obtained in this study may be sufficient to stimulate growth.

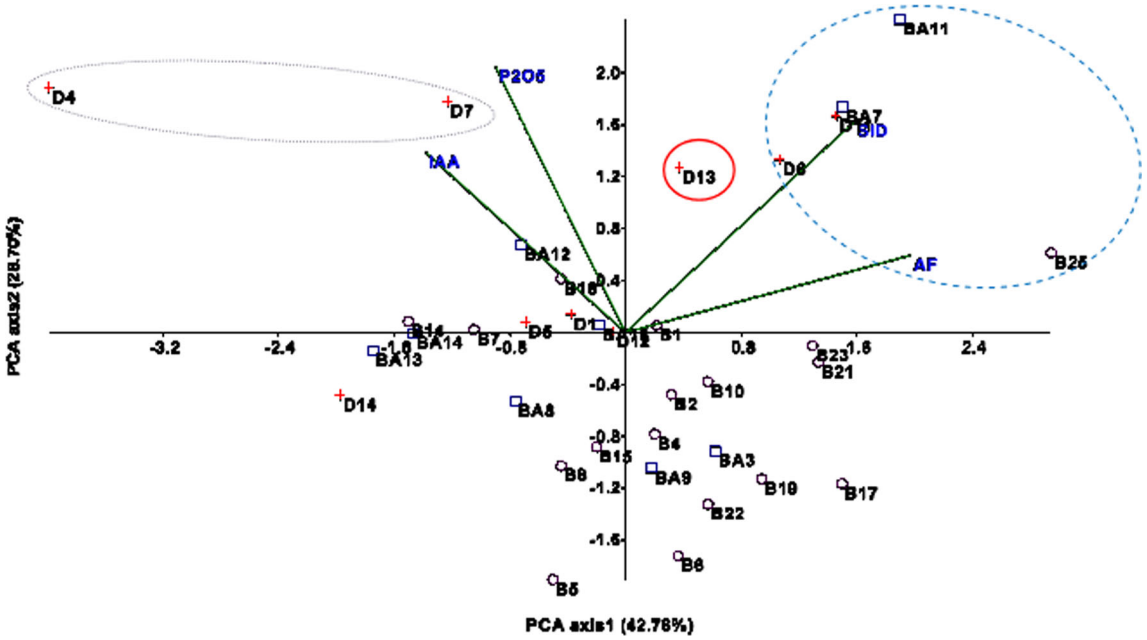

Fig. 4 Principal component analysis (PCA) ordering of data (axes 1 and 2) generated from the PGP activity profiles of the *Bacillus* strains. Groupings were supported by analysis of PGP activity profiles

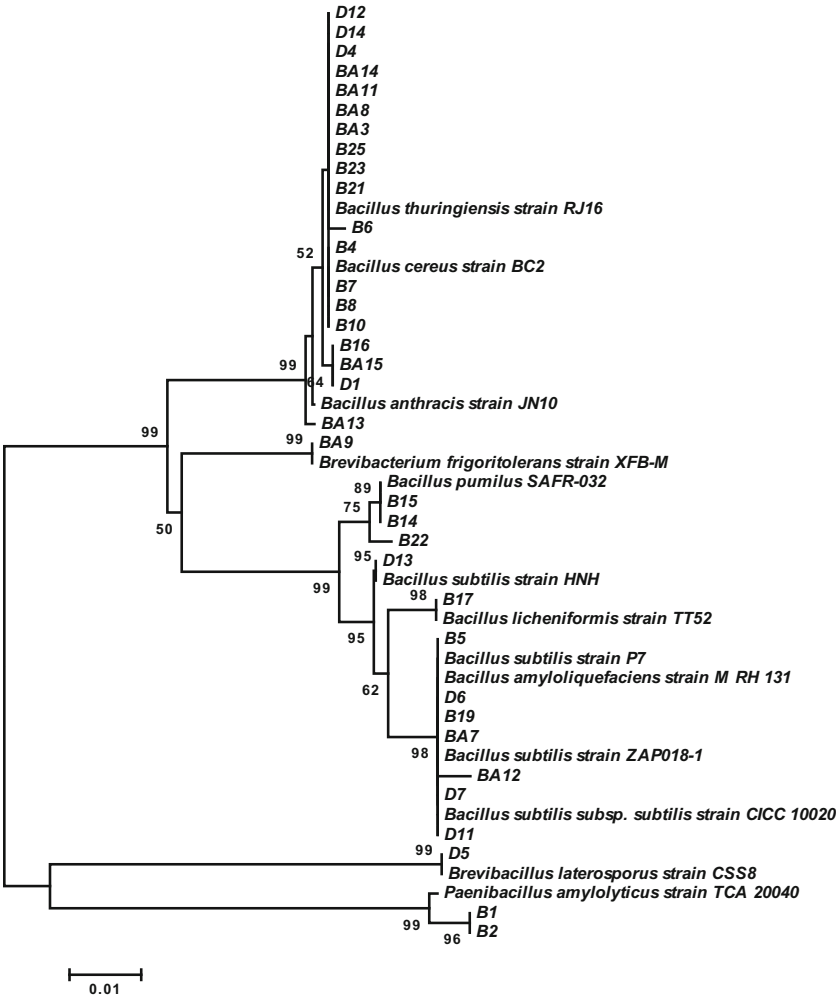

Fig. 5 Phylogenetic tree based on a comparison of the 16S rDNA sequences of *Bacillus* sp. isolates and some of their closest phylogenetic relatives. The tree was created by the neighbour-joining method. The numbers on the tree indicate the percentages of bootstrap sampling derived from 1000 replications. *Bar* Inferred nucleotide substitutions per nucleotide

One of the main *Bacillus* activities enhancing the growth of plants is a mineral supply of phosphate. The application of *Bacillus* as biofertilizer in agriculture has been widely documented. An intrinsic property of *Bacillus* is the ability to solubilise phosphate. *B. polymyxa*, *B. subtilis*, and other species are among the most efficient bacterial communities in terms of phosphate solubilisation (Illmer and Schinner 1992).These bacteria were able to dissolve a quantity of P greater than that necessary for their metabolism, which allowed plants to absorb the surplus (Kloepper et al. 1989). Our results showed that a significant percentage (77 %) of the isolated strains could solubilise phosphates on liquid PVK medium with rates up to 16.65 μg/mL P_2O_5. However, only two strains showed a transparent halo on solid PVK medium. Although most strains of *Bacillus* showed no solubilisation on solid media, they could solubilise phosphate efficiently in liquid medium. Solid media were less sensitive than liquid media in the detection of solubilisation capability. This can be explained by the low diffusion rate of the acids produced by these bacteria in solid medium during their growth. Therefore, an isolate that solubilised $Ca_3(PO_4)_2$ in liquid medium failed to produce a clear zone on solid medium. This suggested that assessing P solubilisation on solid medium is not a reliable technique compared to solubilisation in liquid medium (Nautiyal 1999).

The production of siderophores can influence plant growth by binding iron in its available form: Fe^{+3}. By this process, the iron is made unavailable to plant pathogens. As a result, siderophores protect the health of plants from several fungal or bacterial diseases. Production of siderophores is one of the biological controlling mechanisms belonging to PGPR groups, including *Bacillus* sp., in response to iron-limiting conditions. PGPR produces a range of siderophores that have a strong affinity for iron. The majority of the isolated strains produced siderophores at highly variable rates. This can be explained by the different intrinsic capacity of strains, and environmental factors (pH, temperature and carbon sources) (Valdebinito et al. 2006). The production of siderophores was common to all strains, but on solid medium this production was much less visible than on liquid medium (Tian et al. 2009).

The production of volatile compounds is another mechanism improving plant growth. In *Bacillus*, 2,3-butanediol and acetoin are the compounds most commonly released (Ryu et al. 2003). The latter authors demonstrated that mutant strains of *Bacillus* lacking the gene responsible for production of 2,3-butanediol show no protection against diseases caused by plant pathogens. This confirms the activity of these compounds in inducing resistance against diseases. Of the isolated strains, 56 % were positive for the VP reaction. Thus, they could be potential biocontrol agents. HCN is another volatile product also involved in the suppression of different pathogens. A positive correlation between the production of HCN and the protection of cucumber against *Phytium ultimum* and tomato against *Fusarium oxysporum* is described (Ramette et al. 2003). However, with the exception of D13, the strains tested did not have the capacity to produce HCN. This can be explained by the absence of genes (*hcn*) responsible for the production of this metabolite, or to the absence of a suitable precursor. Glycine and other precursors, such as glutamate or methionine, can be used (Castric 1977).

Concerning antifungal activity, all isolated strains inhibited at least one tested fungal strain and 77 % of these strains had an activity against three or more fungi. *Bacillus* spp. are known for their significant antifungal activity. They produce a variety of powerful antifungal metabolites and hydrolytic enzymes. Several mechanisms have been proposed to explain the inhibition of pathogenic fungi by *Bacillus* spp., including the production of antimicrobial agents, secretion of hydrolytic enzymes, competition for nutrients, or a combination of these mechanisms. Antagonist activity or antibiosis is the best known and most important mechanism used to limit the invasion of the pathogen in the tissues of the host plant. While five strains were inhibitory to all the fungi tested, these results were in agreement with an earlier report that *Bacillus* sp. produced antifungal metabolites with an activity against a number of mycelial fungi (Goswami 2014).

The results of our study showed that the PGP activities of *Bacillus* were heterogeneous. Some strains had remarkable capabilities. Also, they were better phytostimulators than biofertilisers (D4 and D7). Indeed, phosphate solubilisation and the simultaneous production of IAA have been demonstrated, while other strains possessed the power of biocontrol (antifungal activity); B25 illustrated this example. In addition, strains belonging to the same species of *B. thuringiensis* may exhibit different behaviours. However, according to many authors, a highly significant positive correlation between these different activities has been recorded in rhizobacteria (Silini-Cherif et al. 2012).

In addition, the quality of soil has an impact on the expression of these mechanisms. Thus, PGPR have weakly measurable effects on the growth of plants when grown in rich soil under optimal conditions. Indeed, *B. amyloliquefaciens* Bc12 isolated from a semi-arid climate was more effective in improving plants in nutrient-poor soil than in nutrient-rich soil (Egamberdiyera 2007).

The presence of ACC deaminase in four strains of *Bacillus* (B21, B25, D4 and B10) can be used as a tool for selection of these rhizobacteria in promoting plant growth under various environmental conditions. Indeed, this enzyme, by reducing the concentrations of endogenous plant ethylene, can relieve several stress infections, toxicity of heavy metals, high salinity and even drought. ACC deaminase activity was confirmed in many isolates, and the current literature has identified a strong relationship between the rhizobacterial capabilities and colonized plant tolerance to salt stress and can give the plant a certain degree of protection to subsequent attacks by a

phytopathogen via stimulation of systemic defence mechanisms or induced systemic resistance (ISR) (Glazebrook et al. 2003).

The phylogenetic tree based on the 16S rDNA showed that the 15 isolates were divided into 5 different groups representing a relatively low diversity for the rhizospheric *Bacillus*. The majority of our isolates were assigned to *B. subtilis* and *B. thuringiensis*, which form Ash's rRNA group (Ash et al. 1991). This was in agreement with the results of other studies where the percentage of identified species form a distinct clade closely related to the rRNA group 1 of the genus *Bacillus* (Yadav et al. 2011).

It is well known that many different bacterial taxa have been identified as PGPR, but the most commercially developed PGPR are *Bacillus* species that form heat- and desiccation-resistant endospores that confer population stability during the formulation and storage of products. Thus, *Bacillus* spp. may be relatively more versatile than non-spore forming bacteria in the soil (Garbeva et al. 2003). In this study, many different *Bacillus* strains showing multiple PGP attributes were isolated. The results indicated that our isolates could be useful in the formulation of new inoculants.

References

Ahemad M, Kibret M (2014) Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. J King Saud Univ Sci 26:1–20

Amar JD, Kumar M, Kumar R (2013) Plant growth promoting rhizobacteria (PGPR): an alternative of chemical fertilizer for sustainable, environment friendly agriculture. Res J Agric For Sci 1(4): 21–23

Ash CJ, Farrow AE, Wallbanks S, Collins MD (1991) Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small subunit ribosomal RNA sequences. Lett Appl Microbiol 13: 202–206. doi:10.1111/j.1472-765X.1991.tb00608.x

Barazani O, Friedman J (1999) Is IAA the major root growth factor secreted from plant-growth-mediating bacteria. J Chem Ecol 25: 2397–2406. doi:10.1023/A:1020890311499

Cappuccino JC, Sherman N (1992) Microbiology: a laboratory manual, 3rd edn. Benjamin/Cummings, New York, pp 125–179

Castric PA (1977) Glycine metabolism by *Pseudomonas aeruginosa*: hydrogen cyanide biosynthesis. J Bacteriol 130:826–831, PMCID: PMC235287

Ding Y, Wang J, Liu Y, Chen S (2005) Isolation and identification of nitrogen-fixing bacilli from plant rhizospheres in Beijing region. J Appl Microbiol 99:1271–1281. doi:10.1111/j.1365-2672.2005.02738.x

Dworkin M, Foster J (1958) Experiments with some microorganisms which utilize ethane and hydrogen. J Bacteriol 75:592–601, PMCID: PMC290115

Edwards U, Rogall T, Blocker H, Emde M, Bottger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res 17:7843–7853. doi:10.1093/nar/17.19.7843

Egamberdiyera D (2007) The effect of plant growth and nutrient uptake of maize in two different Soils. Appl Soil Ecol 36:184–189. doi:10.1016/j.apsoil.2007.02.005

Erturk Y, Ercisli S, Haznedar A, Cakmakci R (2010) Effects of Plant Growth Promoting Rhizobacteria (PGPR) on rooting and root growth of kiwifruit (*Actinidia deliciosa*) stem cuttings. Biol Res 42:91–98. doi:10.4067/S0716-97602010000100011

Gamalero E, Berta G, Massa N, Glick BR, Lingua G (2010) Interactions between *Pseudomonas putida* UW4 and *Gigaspora rosea* BEG9 and their consequences on the growth of cucumber under salt stress conditions. J Appl Microbiol 108:236–45. doi:10.1111/j.1365-2672.2009.04414.x

Garbeva P, Van Veen JA, van Elsas JD (2003) Assessment of the diversity, and antagonism towards *Rhizoctoniasolani* AG3, of *Pseudomonas* species in soil from different agricultural regimes. FEMS Microbiol Ecol 47:51–64

Gaur AC (1990) Phosphate solubilizing microorganisms as biofertilizers, 1st edn. Omega Scientific, New Delhi, India. ISBN 81-85399-09-3

Glazebrook J, ChenW EB, Chang HS, Nawrath C, Metraux JP, Zhu T, Katagiri F (2003) Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant J 31:217–228, PMID:12694596

Glick BR, Jacobson CB, Schwarze MMK, Pasternak JJ (1994) 1-Aminocyclopropane-1- carboxylic acid deaminase mutants of the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2 do not stimulate canola root elongation. Can J Microbiol 40:911–915. doi:10.1139/m94-146

Goswami D, Dhandhukia P, Patel P, Thakker JN (2014) Screening of PGPR from saline desert of Kutch: growth promotion in Arachis hypogea by *Bacillus licheniformis* A2. Microbiol Res 169:66–75. doi:10.1016/j.micres.2013.07.004

Holl FB, Chanway SP, Turkington R, Radley RA (1988) Response of cresied wheatgrss (*Agropyron cristatum* L.) perennial ryegrass (*Lolium perrenne*) and white clover (*Trifolium repens* L.) to inoculation with *Bacillus polymyxa*. Soil Biol Biochem 20:19–24, **http://dx.doi.org/10.1016/0038-0717(88)90121-6**

Illmer P, Schinner F (1992) Solubilization of inorganic phosphates by microorganisms isolated from forest soil. Soil Biol Biochem 24: 389–395, **http://dx.doi.org/10.1016/0038-0717(92)90199-8**

Jacobson CB, Pasternak JJ, Glick BR (1994) Partial purification and characterization of 1-aminocyclopropane-1-carboxylate deaminase from the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. Can J Microbiol 40:1019–1025, **http://dx.doi.org/10.1139/m94-162**

Joseph B, Patra RR, Lawrence R (2007) Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). Int J Plant Prod 2:141–152

Kloepper JW, Litshitz R, Zablotowicz RM (1989) Free living bacterial inocula for enhancing crop producitivity. Trends Biotechnol 7:39–43. doi:10.1016/0167-7799(89)90057-7

Kumar NR, Arasu VT, Gunasekaran P (2002) Genotyping of antifungal compounds producing plant growth-promoting rhizobacteria *Pseudomonas fluorescens*. Curr Sci 82:1463–1466

Loper JE, Schroth MN (1986) Influence of bacterial sources of indole-3-acetic acid on root elongation of sugar beet. Phys Chem Chem Phys 76:386–389, **http://dx.doi.org/10.1094/Phyto-76-386**

Lorck H (1948) Production of hydrocyanic acid by bacteria. Physiol Plant 142-146 doi: 10.1111/j.1399-3054.1948.tb07118.x

Maplestone PA, Campbell R (1989) Colonization of roots of wheat seedlings by bacilli proposed as biocontrol agents against take-all. Soil Biol Biochem 21(4):543–550. doi: **10.1016/0038-0717(89)90128-4**

Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:260–270

Olsen SR, Sommers LE (1982) Phosphorus. In: Page AL, Miller RH, Keeney DR (eds) Methods of soil analysis, part 2, chemical and microbial properties, 2nd edn. American Society of Agronomy, Madison, pp 403–430

Qiao JQ, Wu HJ, Rong Huo R, Gao XW, Borriss R (2014). Stimulation of plant growth and biocontrol by *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 engineered for improved action. Chemical and Biological Technologies in Agriculture 1:12

Ramette A, Frapolli M, Defago G, Moenne-Loccoz Y (2003) Phylogeny of HCN synthase-encoding *hcn* BC genes in biocontrol fluorescent pseudomonads and its relationship with host plant species and HCN synthesis ability. Mol Plant-Microbe Interact 16:525–535. doi:10.1094/MPMI.2003.16.6.525

Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Paré PW, Kloepper JW (2003) Bacterial volatiles promote growth in *Arabidopsis*. Proc Natl Acad Sci USA 100:4927–4932. doi:10.1104/pp.103.026583

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425, PMID: 3447015

Shwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Annu Rev Plant Physiol Plant Mol Biol 160:47–56

Silini-Cherif H, Silini A, Ghoul M, Yadav S (2012) Isolation and characterization of plant growth promoting traits of a rhizobacteria: *Pantoea agglomerans* lma2. Pak J Biol Sci 15:267–276. doi:10.3923/pjbs.2012.267.276

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739. doi:10.1093/molbev/msr121

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 11(22):4673–4680, PMID: 7984417

Tian F, Ding Y, Zhu H, Yao L, Du B (2009) Genetic diversity of siderophore-producing bacteria of tobacco rhizosphere. Braz J Microbiol 40:276–284. doi:10.1590/S1517-83822009000200013

Vacheron J, Desbrosses G, Bouffaud M-L et al (2013) Plant growth-promoting rhizobacteria and root system functioning. Front Plant Sci 4:356. doi:10.3389/fpls.2013.00356

Valdebinito M, Crumbliss AL, Winkelman G, Hantke K (2006) Environmental factors influence the production of enterobactin, salmochelin, aerobactin and yersiniabactin in *Escherichia coli* strainNissle 1917. Int J Med Microbiol 296:513–520, PMID: 17008127

Wilson K (1990) Preparation of genomic DNA from bacteria. In: Ausubel FM, Brent R (eds) Current protocols in molecular biology. Greene/Wiley Interscience, New York, pp 241–245

Yadav S, Kaushik R, Saxena AK, Arora DK (2011) Diversity and phylogeny of plant growth-promoting bacilli from moderately acidic soil. J Basic Microbiol 51(1):98–106. doi:10.1002/jobm.201000098

Young JPW, Downer HL, Eardly BD (1991) Phylogeny of the phototrophic *Rhizobium* strain BTAil by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. J Bacteriol 173:2271–2277, PMCID: PMC207778

Zahir ZA, Arshad M, Frankenberger WT (2004) Plant growth promoting rhizobacteria: applications and perspectives in agriculture. Adv Agron 81:97–168. doi:10.1016/S0065-2113(03)81003-9