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Growth promotion of rapeseed (*Brassica napus*) associated with the inoculation of phosphate solubilizing bacteria

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ABSTRACT

Inoculation of microorganisms to improve crop yields and minimize the use of chemical fertilizers is a promising sustainable strategy. The objective of this study was to isolate and identify bacteria from the rhizosphere of rapeseed plants able to solubilize phosphate and promote the growth of this crop. We isolated 40 different bacterial morphotypes which were phenotypically and genotypically characterized. Fourteen isolates (37.8%) were able to solubilize phosphate, 9 of them being epiphytic and 5 endophytic. In greenhouse experiments 7 of these isolates increased shoot dry weight, reaching values similar to those of fertilized plants. Additionally, we determined that survival, growth and biofilm formation ability of these bacteria were not affected by rapeseed root exudates. All the phosphate solubilizing strains able to promote plant growth under greenhouse conditions, with the exception of *Bacillus* sp. LTAD-52, also increase rapeseed yield (from 21 to 44%) in field trials.

1. Introduction

The rhizosphere is that part of soil affected by plant roots (Hinsinger et al., 2009). Many microorganisms reside there and bacteria are the most abundant among them. Rhizosphere bacteria able to colonize plant roots are potentially useful to influence plant health and growth, increasing crop yields. These bacteria are collectively referred to as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al., 1989). They can benefit plant growth directly by either providing essential nutrients (phosphorus, nitrogen and essential minerals) or by mimicking synthesis of plant hormones, or indirectly by preventing the deleterious effects of phytopathogenic organisms through production of antibiotics and siderophores or induction of systemic resistance in the host plant (Renwick et al., 1991; Pal et al., 2001; Karthikeyan et al., 2005; Romero et al., 2007).

To grow and persist, bacteria should be able to effectively colonize root or rhizosphere and compete with other organisms (Dennis et al., 2010). Compounds secreted by plant roots into the soil (generally called root exudates) may regulate the soil microbial structure and function, by changing soil chemical and physical properties (Vessey, 2003; Bais et al., 2006; Hartmann et al., 2009). Plant developmental stage and species, and also biotic and abiotic factors may impact on the quantity and quality of root exudates (Bais et al., 2006). Low-molecular-weight compounds in root exudates consist of amino acids, organic acids, sugars, phenolics and secondary metabolites, whereas mucilage and proteins are examples of high-molecular-weight compounds (Walker et al., 2003).

Prior to root colonization (the first step required to plant growth promotion), chemotaxis of PGPR towards the roots system is required (Sood, 2003; Zheng and Sinclair, 1996; Kumar et al., 2007; Tan et al., 2013). PGPR colonize the root surfaces and proliferate by receiving key signaling compounds and nutrients released by the plant, which often lead to the formation of biofilm communities (Badri and Vivanco, 2009). Biofilm formation on the roots is indicative of successful PGPR colonization, and biofilm organisms are significantly more resistant to environmental stresses or microbial deleterious substances (such as antibiotics and biocides) than planktonic cells (Annous et al., 2005; Lugtenberg et al., 1999; Walker et al., 2004; Shi et al., 2012). In some bacteria biofilm formation is a process controlled by a cell-to-cell communication, known as quorum sensing (Costerton et al., 1978). This phenomenon is mediated by chemical autoinducer molecules released by bacteria. Gram-negative bacteria use the acyl-homoserine lactone (AHL) as autoinducer whereas Gram-positive bacteria utilize modified peptides as autoinducer molecules (Waters et al., 2008; Ng and Bassler, 2009; Brameyer et al., 2015).

Numerous PGPR have been assayed as biofertilizers at field, considering that they are able to provide inorganic nutrients to plants (Mendez-Castro and Alexander, 1983). After nitrogen, phosphorus (P)

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is the most important mineral macronutrient required by plants. In soils this element occurs as organic and mineral forms, and is absorbed by plants as phosphates (Subba Rao, 1988). However, P combines with other soil materials and these combined forms limit P movement in soils. Therefore, this nutrient becomes unavailable to plant root system, even when its concentration in some soils is high (Alexander, 1980). Inorganic phosphates applied as chemical fertilizers can also be immobilized in the soil and consequently to be not available to crops (Peix et al., 2001). Therefore, phosphate-solubilizing bacteria (PSB) may play an important role in the plant P nutrition. Various studies have demonstrated that inoculation with PSB results in higher crop yields (Chabot et al., 1996; Pal, 1998; Wakelin et al., 2004; Hameeda et al., 2008; Babana and Atoun, 2006; Ahmed and El-Araby, 2012). Seneviratne and Jayasinghearachchi (2005) reported that application of PSB directly into soil increased P availability by 15-fold and that the biofilmed inocula can be effectively used in biosolubilization of rock phosphate. In this sense, rapeseed has been little studied even though it is the second most important oilseed crop in the world after soybean (Hall et al., 1996; Bertrand et al., 2001, Salimpour et al., 2010). Considering the importance of using PSB to improve crop yields, minimizing the use of chemical fertilizers, the objectives of this study were to isolate and identify PSB from the rhizosphere of rapeseed plants, and to evaluate the influence of rapeseed root exudates on their survival, growth and biofilm formation ability. Additionally, the capacity of these PSB to promote rapeseed plant growth in a field assay was determined.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The isolates obtained in this work, as well as *Serratia* sp. S119 and *Pantoea* sp. J49 (Taurian et al., 2010), *Klebsiella* sp. NTI-31 (Ibáñez et al., 2009), *Azospirillum brasilense* Cd (EMBRAPA, Brasil), *Pseudomonas fluorescens* (Rizofost®) and *Pseudomonas aureoginosa* PAO1 (Holloway, 1955) were grown at 28 °C in trypticase soy agar medium (TSA, Britania). *Chromobacterium violaceum* CV026 (Winson et al., 1995) was cultured in LB medium (Miller, 1992) supplemented with kanamycin (Km; 50 μg ml^{-1}) at 28 °C. *Agrobacterium tumefaciens* NT1-pZLR4 (Cha et al., 1998) was cultured in YEM medium (Vincent, 1970) supplemented with gentamicin (Gm; 30 μg ml^{-1} at 28 °C. *Sinorhizobium fredii* HH103 (Buendía-Clavería et al., 1989) was grown at 28 °C in YEMA (Yeast Extract Mannitol Agar) medium (Vincent, 1970). The strains were kept in 20% glycerol at −80 °C for long-term storage and in 40% glycerol at 20 °C for short-term storage.

2.2. Isolation of rapeseed epiphytic and endophytic bacteria

They were isolated from plants growing at fields located in Rio Cuarto (Córdoba, Argentina) and Tres Arroyos (Buenos Aires, Argentina). For the isolation of epiphytic bacteria, roots were separated, washed with sterilized water and resuspended in buffered saline solution (PBS, NaCl 0.14 M; KCl 0.0027 M; Na_2HPO_4 0.01 M; KH_2PO_4 0.0018 M, pH 7.4). Dilutions of this solution were spread on plates containing 10% TSA medium supplemented with 50 μg ml^{-1} of cycloheximide (to control fungal growth) and the plates were incubated at 28 °C for 7 days (Kuklinsky-Sobral et al., 2004). For the isolation of endophytes, epiphytes were previously removed by surface disinfection of plant root using serial washing in 70% ethanol for 1 min, 2% sodium hypochlorite for 3 min, 70% ethanol for 30 s and two rinses in sterilized distilled water. The success of the disinfection process was checked by plating aliquots of the sterile distilled water used in the final rinse onto 10% TSA and incubating the plates at 28 °C. Dilutions of this solution were plated on 10% TSA medium supplemented with 50 μg ml^{-1} of cycloheximide and the plates were incubated at 28 °C for 7 days (Kuklinsky-Sobral et al., 2004). Morphological and staining properties of bacteria from different colonies were determined.

2.3. Identification of phosphate solubilising isolates

In vitro, inorganic phosphate-solubilizing ability was determined by using NBRIP-BPB solid medium (National Botanical Research Institute's phosphate grown medium) (Mehta and Nautiyal, 2001) containing tricalcium-phosphate as sole source of P. Ten microliters of fresh bacterial culture (10^8 CFU ml^{-1}) were spotted onto these plates and incubated at 28 °C for 7 days. The halo of clearance around the bacterial colony indicated solubilizing ability.

2.4. Evaluation of rapeseed growth promotion by native PSB isolates

Isolates able to solubilize phosphates were evaluated on their ability to promote plant growth. Seeds used for test in plant growth chamber were commercial spring variety BIOLZA 440. They were sterilized in sodium hypochlorite 20% for 5 min and washed 5 times with sterile distilled water, and germinated at 28 °C in sterilized Petri dishes with one layer of Whatman N°1 filter paper and moist cotton. When radicles reached 1 mm long they were transferred to pots containing 360 cm^3 sterilized volcanic sand, supplemented with 40 ml of $CO_3(PO_4)_2$ 0.2%, and watered with phosphorus-free Hoagland solution (Hoagland, 1950) every 3 days during 5 weeks. Plants were grown in chamber under controlled environmental conditions (Conviron MTPS, light intensity of 200 μE m^2 s^{-1}, 18-h day/6-h night cycle, at a constant temperature of 25 °C and a relative humidity of 60%). One week after sowing, plants were inoculated with 1 ml of bacterial cultures in TSB medium, which previously was harvested by centrifugation, washed and resuspended in saline solution (NaCl 0,9%) in order to obtain an $O.D._{620} = 1$ (10^9 CFU ml^{-1}). Each treatment was plot with five repetitions and replicated twice. Seedlings inoculated with *Pseudomonas fluorescens* (phosphate solubilizer strain used in commercial maize and wheat inoculant formulation, Rizofos® – Rizobacter) and non-inoculated seedlings grown under the same conditions (negative control) were included. Plants growing in volcanic sand without the addition of $CO_3(PO_4)_2$ and watered with Hoagland solution (Hoagland and Arnon, 1950) containing 1 mM of KH_2PO_3were used as positive control. Four weeks after inoculation (rosette stage), rapeseed plants were harvested and their fresh and dry aerial weights and number of developed leaves were determined.

2.5. PCR amplification and partial sequencing of the 16S rRNA gene from PSB

PSB that showed plant growth promotion were identified by the sequence of their 16S rRNA genes. Partial amplification of this gene was performed by using primers 518F (5′-CCAGCAGCGGTAATACG-3′) and 800R (5′-TACCAGGGTATCTAATCC-3′). Amplification product (1500 bp) was sent to Macrogen Laboratories Inc. (Korea) for sequencing. Analysis of the sequences obtained was performed using the BLASTN algorithm (Altschul et al., 1997).

2.6. Collection of root exudates

Ten seedlings growing in sterilized Petri dishes as previously described were individually transferred to 360 cm^3 glass beakers containing 100 ml sterile distilled water covered with aluminum foil. Radicle (1 cm long) were passed through an aluminum foil hole and ensured that only this organ was submerged in the water (Taurian et al., 2008). Plants were grown in a chamber with controlled environmental conditions. After 7 days of incubation, the solution bathing roots, termed root exudates, was collected. Sterile root exudates were centrifuged at 6200 rpm for 15 min and filtered through 0.8 μm and 0.2 μm polycarbonate filters. The sterile root exudates were used to prepare TSB medium, with a reduced concentration (10%) of its components (it

was called TSBr10%). This culture medium was sterilized by filtration through nitrocellulose membranes 0.2 μm pore diameter.

2.7. Bacterial growth in presence of rapeseed root exudates

Colonies of bacteria grown for 24 h at 28 °C in Petri dishes containing TSA medium were transferred to TSB and incubated on a rotary shaker (180 rpm) at 28 °C for 18 h. Bacterial culture volume required to obtain an initial $O.D._{620nm}$ = 0.1 was inoculated in 20 ml TSBr10% prepared with or without root exudates. Cultures were incubated at 28 °C in a rotary shaker at 180 rpm and growth was evaluated spectrophotometrically by determining the absorbance at 620 nm every 2 h. At 24 h of incubation, bacterial survival was evaluated following the method described by Somasegaran and Hoben (1994). Plates were incubated in an oven at 28 °C for 18 h, and the number of CFU ml^{-1} was determined.

2.8. Biofilm formation assay

The biofilm formation assay, based on the method of O'Toole and Kolter (1998), relies on the ability of cells to adhere to the wells of 96-well polyvinylchloride microtiter dishes. Two hundred μl of a 1:100 dilution of an overnight culture ($O.D._{620nm}$ = 0.2) in TSBr10%, with or without root exudates, was added to each well. The plates were covered with plastic to prevent evaporation and incubated in a rotary shaker (180 rpm) at 28 °C for 48 h. Planktonic cells were gently homogenized manually by repeated pipetting and bacterial growth was determined by measuring O.D. at 600_{nm}. Cultures were aspirated using an automatic hand pipette, and wells were washed three times with 180 μl of PBS and stained for 15 min with 150 μl of 0.1% crystal violet solution (CV). Each CV-stained well was then rinsed thoroughly and repeatedly with water, and scored for biofilm formation by addition of 150 μl of ethanol 95%. The O.D. at 560_{nm} of solubilized CV was determined using a MicroELISA Auto Reader (Series 700 Microplate Reader, Cambridge Technology). In order to normalize the absorbance values obtained in relation to the number of bacteria contained in each well, the biofilm formation index (BI) was calculated by the following formula: $IB = O.D._{560nm}/O.D._{600nm}$.

2.9. Preparation of AHLs extracts

Extraction of AHLs was performed as described by Eberhard et al. (1981). Isolates were grown in 20 ml TSBr10% (with or without root exudates) on a rotary shaker (180 rpm) for 48 h at 28 °C. The cultures were centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatants were extracted three times with 20 ml of acidic ethyl acetate (0.1 ml glacial acetic acid per liter). The extracts were dried, resuspended in 100 μl of ethyl acetate and stored at −80 °C for autoinducer detection.

2.10. Diffusion plate assays

Biosensors *C. violaceum* CV026 (Winson et al., 1995) and *A. tumefaciens* NTL4 (pZLR4) (Cha et al., 1998) were used to detect AHLs with short (C4 to C8) or long acyl chains (C6-C12), respectively. *C. violaceum* CV026 is a mini Tn5 double mutant defective in the synthesis of violacein pigment. The *in vitro* production of this pigment is activated by AHLs with short acyl chains. These autoinducers were detected by the method described by Mc Clean et al. (1997). *C. violaceum* CV026 was grown overnight in LB broth (supplemented with Km 50 μg ml^{-1}) to an $O.D._{620nm}$ = 1. A sample (1.6 ml) of this culture was added to 40 ml of LB containing molten agar (0.8%) at 50 °C. Five ml of this soft agar suspension was poured onto pre-warmed Petri dishes containing LB agar, and 5 μl AHL extracts were poured. Plates were incubated at 28 °C for 48 h and the diameters of the halo formed were measured. *A. tumefaciens* NTL4 (pZLR4) carries the plasmid pZLR4, which contains a *traG*::*lacZ* fusion and traR. In the presence of long-chain AHLs, the *TraR* protein is activated, transcription of the *traG*::*lacZ* fusion is turned on, and LacZ (β-galactosidase) activity is measure as a reporter of *traG* transcription. AHL-like molecules with long acyl chains were detected by the method of Farrand et al. (2002). *A. tumefaciens* NTL4 was grown overnight in YEMA broth medium (supplemented with Gm 30 μg ml^{-1}) to an $O.D._{620nm}$ = 1.0. A 4 ml sample of this culture was added to 40 ml of LB containing molten agar (0.8%) at 50 °C and supplemented with 400 μl Xgal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside, 20 mg ml^{-1} in dimethylformamide) and 16 μl Gm (80 μg ml^{-1}). Five ml of this soft agar suspension was poured onto pre-warmed Petri dishes containing YEMA medium and 5 μl AHL extract samples were poured. The plates were incubated at 28 °C for 48 h and the diameters of halo formed were measured. *P. aeruginosa* PAOI y *S. fredii* HH103 strains were used as positive controls for AHL short chain (C4-C6) and long chain (C10-C18) production, respectively.

2.11. Field assays

PSB that showed rapeseed plant growth promotion in growth chamber were selected to evaluate their behaviour in field trials. Twenty five ml of bacterial cultures in TSB medium ($O.D._{620nm}$ = 1.0) were transferred to 500 ml TSB medium and incubated at 28 °C on a rotary shaker (180 rpm). When cultures reached an $O.D._{620nm}$ (10^9 CFU ml^{-1}) were transferred to a sterile bladder (250 ml) and stored at 4 °C until use. To evaluate field performance of inoculants, an assay was carried out at an experimental farm in Barrow (38°19′13.4″S 60°14′31.8″W), INTA, Argentina, in 2013 season. At the beginning of the experiment, physical and chemical soil properties were determined, according to standard methods (Pavan et al., 1992) (Table 1). The experimental soil belongs to silt loam typic argiudoll. The average annual rainfall in this region is 750 mm. Rapeseed seeds commercial cultivar spring variety Bioaureo 2386 were planted in a conventional tillage system, with a seeding rates of 80 seeds m^2 and a planting depth of 2.5 cm in a completely randomized block design with three replicates. The size of the plots was 8 rows 6 m long separated by 32 cm. Before sowing, seeds were inoculated (800 ml 100 kg $seed^{-1}$). Treatments were: a) negative control (P fertilizer not applied and non-inoculated), b) positive control (fertilized with diammonium phosphate, 90 kg ha^{-1} uninoculated) and c) PBS inoculated and unfertilized. Weather conditions during the growing season were monitored with a meteorological station located less than 100 m away from the research plot. Monthly precipitation (55.1 mm), mean air temperature (11.8 °C) were adequate. During the growing season (July to December) plants obtained from 1 m^2 were evaluated in the following variables: a) dry matter

Table 1
Soil physical and chemical properties.

Depth cm	Organic Matter %	NO_3-N mg kg^{-1}	Humidity %	Phosphorus mg kg^{-1}	Sulfur mg kg^{-1}	SO_4-S mg kg^{-1}	pH
0–20	2.9	59.6	5.0	32.2	312.6	17.1	5.7
20–40	ND†	40.2	ND†	ND†	ND†	13.5	ND†
40–60	ND†	16.6	ND†	ND†	ND†	12.9	ND†

† **ND:** Not determined.

production at the phenological stages rosette (B6), flowering (F) and ripening (G), b) leaf content of phosphorus (Roseta B6). At harvest (ripening), plants obtained from 3.58 m^2 were evaluated in the following variables: a) grain yield per ha; b) 1000 grain weight; and, c) harvest index (H.I.) (the ratio of grain dry weight to above-ground dry weight at harvest maturity). To obtain dry weight, plant tissues were dried in a forced-air drying oven at 60 °C, to a constant weight. The P content was determined by the colorimetric method described by Murphy and Riley (1962). Yield parameters were evaluated according to Taheri et al. (2012). The typical cultural practices for the rapeseed production were applied. The experimental plots were not irrigated and, therefore, growth was conditioned by cumulative rainfall.

2.12. Statistical analysis

Data were subjected to Analysis of variance (ANOVA), followed by comparison of treatment means, using Least Significant Difference (LSD) at 5% level of significance. Statistical analyses were performed using Infostat software version 2014 (Di Rienzo et al., 2014). The assumptions of variance analysis were tested by insuring that the residuals were random, homogenous, with a normal distribution about a mean of zero.

3. Results and discussion

3.1. Phosphorus solubilizing bacteria identification

A total of 40 different bacterial isolates were identified (19 endophytes and 21 epiphytes) according to their morphological and staining characteristics, and confirmed by their ERIC-PCR profiles (data not shown). All of them were screened in their phosphate solubilizing ability in NBRIP-BPB (Mehta and Nautiyal, 2001) medium. Fourteen isolates (37.8%) showed this ability being 9 of them epiphytic and 5endophytic. Diameter of phosphate solubilization halo ranged from 0.5 to 8.8 mm. The epiphytic isolates LRCP-17, LRCP-29 and LRCP-37 formed the largest diameters (Fig. 1). Facultative endophytic and also epiphytic bacteria might be able to produce many metabolites involved in defense, as they have to compete in the rhizosphere. Instead of that, these metabolites are not essential for obligate endophytic bacteria but they may produce other specific metabolites needed for the interaction with the host. Irrespective of their plant-associated lifestyle, soil bacteria may strongly influence the growth and the biotic and abiotic stress tolerance of plants (Brader et al., 2014). Therefore, we continue our studies analyzing both epiphytic and endophytic phosphorus solubilizing isolates.

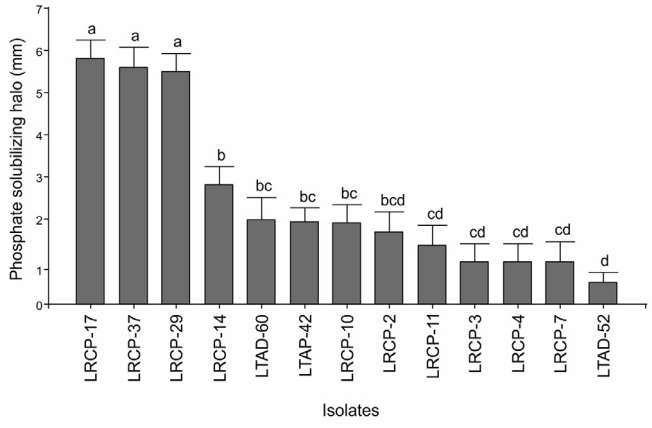

Fig. 1. Phosphate solubilizing halo. Data represent the mean ± SE of two independent replicates with three repetitions each. Values followed by the same letters are not significantly different at $P \leq 0.05$, for each parameter evaluated.

3.2. Effect of phosphate solubilizing bacteria inoculation on plant growth

Phosphate solubilizing isolates were inoculated in rapeseed plants to determinate their capacity to promote plant growth in greenhouse trials. In this assay, the PSB *Klebsiela* sp. NTI31, *Serratia* sp. S119 and *Serratia* sp. J49 isolated from peanut plants (Taurian et al., 2010) and *Pseudomonas fluorescens* (used as commercial inoculant for wheat and maize), were used as reference strains. At four weeks after inoculation, plants were harvested. A significant increase in shoot fresh weight was observed in plants inoculated with 13 of the evaluated isolates, compared with fertilized plants. However, only 7 of these isolates increased shoot dry weight, reaching values similar to those of fertilized plants, and higher than uninoculated plants (negative control) (Fig. 2).

Fertilized and uninoculated plants developed the same number of leaves without differences in the time taken to reach the rosette stage. However, plants inoculated with 15 of the evaluated PSB showed a significant increase in this parameter compared to uninoculated plants. Fast rosette development encourages rapeseed root growth, reduces soil moisture evaporation and shades weeds (Iriarte and Valetti, 2008). Previous reports also demonstrated that PGPR inoculation increases the growth of rapeseed. Kloepper et al. (1988) and Bertrand et al. (2001) reported rapeseed growth promotion by inoculation of endophytes belonging to the genus *Pseudomonas*. de Freitas et al. (1997) found that inoculation of *Bacillus* sp. and *Xanthomonas maltophilia* isolated from rapeseed rhizosphere had positive effects on plant growth, but not on plant P content.

3.3. Taxonomical identification of PSB

Partial sequence (1500 bp) of 16S rRNA gene from those PSB that increased rapeseed growth was analyzed. Bacteria identified in this study belong to *Arthrobacter* sp. (LRCP-11), *Serratia* sp. (LRCP-29), *Pantoea* sp. (LRCP-17) and *Bacillus* genus (LRCP-2, LRCP-3, LRCP-4, LTAD-52) (Table2). This is the first report demonstrating the rapeseed growth promotion by bacteria belonging to these genera.

3.4. Effect of rapeseed root exudates on PSB growth survival, biofilm formation and production of quorum sensing signals

Plant roots exude a wide variety of chemical compounds. Root exudate compounds mediate plant–microbe and microbe–microbe interactions, affecting the rhizomicrobiome diversity. Components of root exudates such as carbohydrates, organic acids, and amino acids stimulate bacterial chemotactic responses and influence the structure and composition of plant associated microbial communities (Somers et al., 2004; Hartmann et al., 2009). Some exudates may also impact negatively on the bacterial colonization (Bais et al., 2006). In turn, microorganisms associated with plantscan influence their health and growth.

In this work, the effect of root exudates on survival and growth of PSB able to increase biomass of rapeseed plants was determined. With the exception of *Serratia* sp. LRCP-29, whose growth was slightly but significantly stimulated after 12 h of incubation, bacterial growth and survival were not affected by the addition of root exudatesto the culture medium (Fig. 3, Table 3). Therefore, we suggest that there are no compounds in rapeseed exudates that would negatively affect the growth and viability of these PSB.

Biofilm formation is crucial for efficient root colonization (Bais et al., 2004. de Weert et al., 2002, Yuan et al., 2015) and constitutes a strategy for bacterial survival in different environments (Ramey et al., 2004; Webb et al., 2003). The ability to form biofilm differed among isolates tested. *Bacillus* sp. LRCP-3 showed the highest rate while *Serratia* sp. LRCP-29 showed the least ability to form biofilm (Table 3). Superior ability to develop biofilm, provides the bacteria an advantage to establish in a given environment, colonize the rhizosphere and compete with other microorganisms (Hamon and Lazazzera, 2001; Molina et al., 2003; Danhorn and Fuqua, 2007). Therefore this property

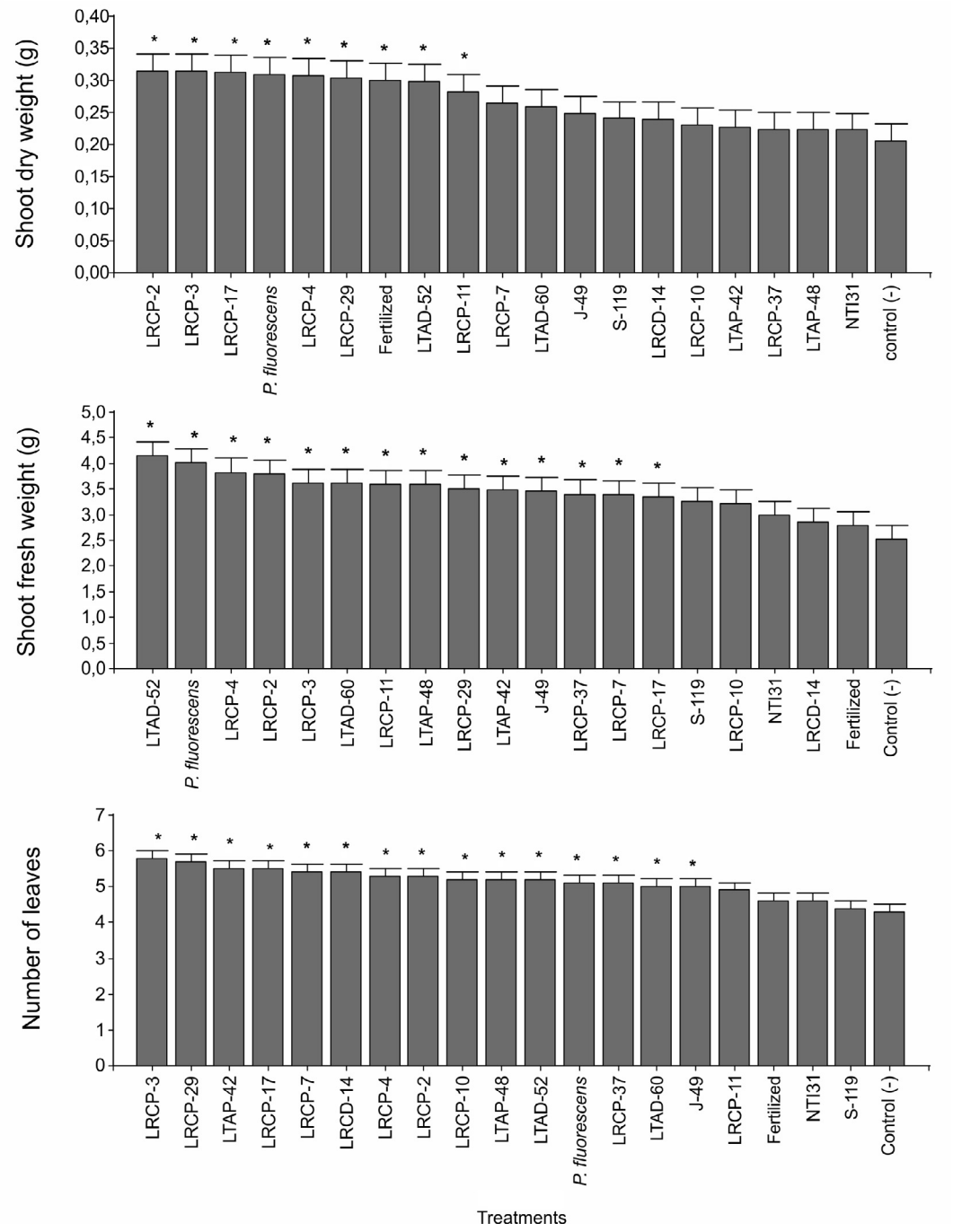

Fig. 2. Effect of PSB inoculation on plant growth. Data represent the mean ± SE of two independent replicates with five repetitions each. *Value is significantly different ($P \leq 0.05$) respect to negative control.

Table 2
PSB identification by 16S rDNA sequencing.

Isolates	GenBank identity	Gene Identity	Cover Sequence	Bacterial Group
LRCP-11	*Arthrobacter* sp PO-08 (AB259962.1)	99	100	Actinobacteria
LRCP-17	*Pantoea agglomerans* ChDC YP1 (AY691543.1)	99	99	Proteobacteria
LRCP-29	*Serratia nemathodiphila* P36 (FJ662869.1)	99	99	Proteobacteria
LRCP-3	*Bacillus safensis* BN-2 (AB971370.1)	99	100	Firmicutes
LRCP-4	*Bacillus* sp. RKAT-07 (LN849695.1)	99	100	Firmicutes
LRCP-2	*Bacillus megaterium* KNUC9061 (JF505995.1)	99	100	Firmicutes
LTAD-52	*Bacillus pumilus* MUST-3 (KF727586.1)	99	100	Firmicutes

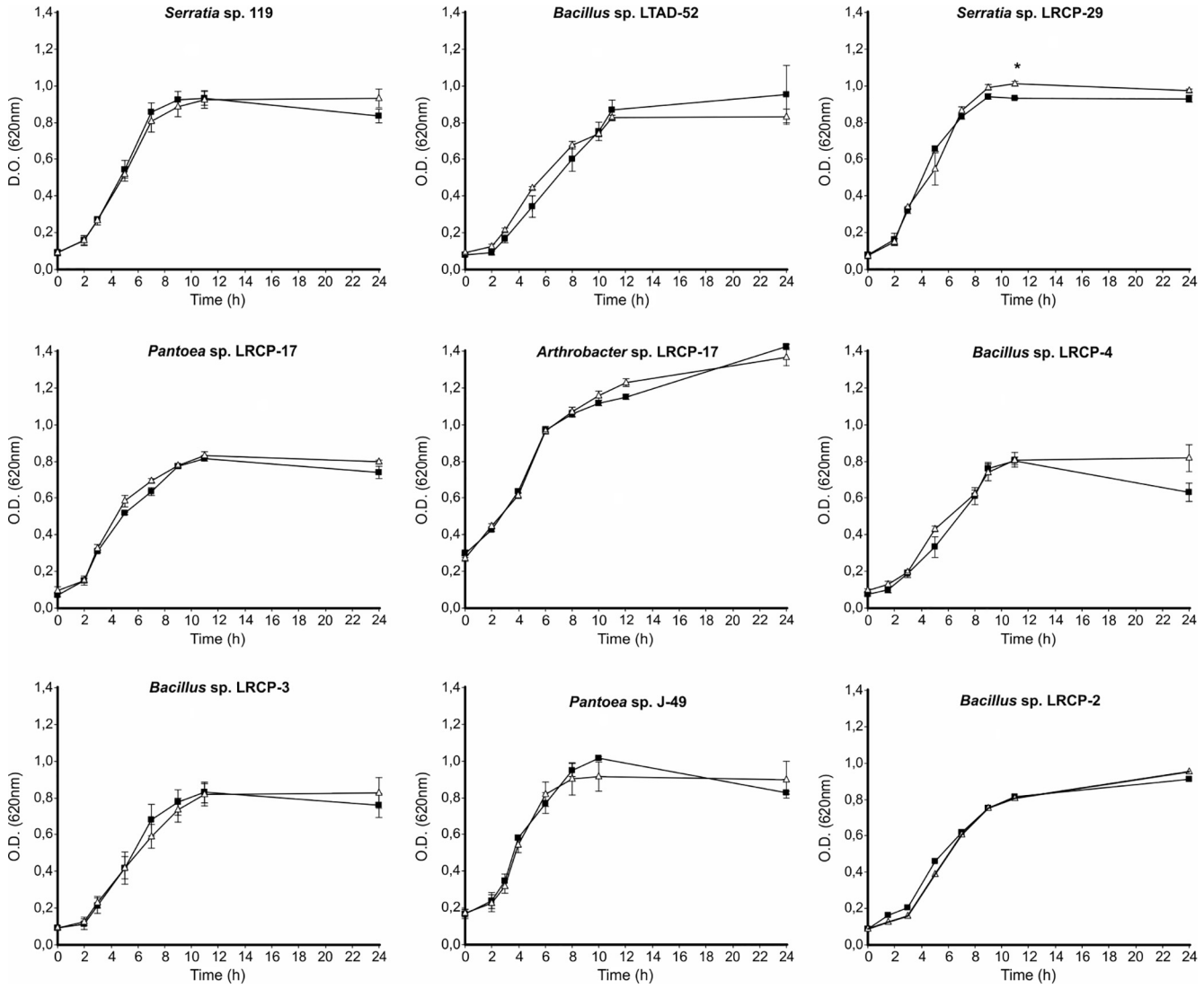

Fig. 3. Effect of rapeseed root exudates on PSB growth. Isolates were grown in TSBr10% medium (black squares) or in TSBr10% medium with root exudates (white triangle). Data represent the mean ± SE of two independent replicates with three repetitions each. *Value is significantly different ($P \leq 0.05$).

Table 3
Effect of rapeseed root exudates on PSB survival and biofilm formation.

Isolates	Survival		Biofilm formation index†	
	With exudates CFU ml^{-1}	Without exudates CFU ml^{-1}	With exudates	Without exudates
Bacillus sp. LTAD-52	1.14E^{7}	1.14E^{7}	8.74b	8.42c
Bacillus sp. LRCP-2	6.44E^{4}	6.88E^{4}	9.25b*	12.35b
Bacillus sp. LRCP-3	8.64E^{6}	6.98E^{6}	21.36a	20.70a
Bacillus sp. LRCP-4	9.80E^{6}	2.96E^{6}	5.41c	5.25d
Arthrobacter sp. LRCP-11	3.76E^{7}	2.31E^{7}	3.50d*	0.13e
Pantoea sp. LRCP-17	3.14E^{7}	3.66E^{7}	2.87de*	5.44d
Serratia sp. LRCP-29	5.77E^{7}	8.44E^{7}	0.53f*	0.96e
Serratia sp. S119	0.91E^{8}	1.40E^{8}	1.59ef*	1.95e
Pantoea sp. J49	2.99E^{7}	3.33E^{7}	6.25c*	11.11b

† Values within a row followed by the same letter are not significantly different at P ≤ 0.05.

* Value is significantly different at $P \leq 0.05$ from others in the same row.

could be relevant when selecting isolates to assess their behavior in field trials.

The root exudates can stimulate or inhibit the formation of biofilm (Bais et al., 2006; Teplistki et al., 2000). Our results indicate that root exudates significantly promoted the biofilm formation of 5 isolates (*Pantoea* sp. J49, *Bacillus* sp. LRCP-2, *Serratia* sp. S119, *Pantoea* sp. LRCP-17 and *Serratia* sp. LRCP-29) while they decreased this ability of only one of them (*Arthrobacter* sp. LRCP-11) (Table 3). It has been reported that exudates from rice and bean seeds stimulate biofilm formation of *S. fredii* SMH12 and, therefore, the root colonization, while rice root exudates did not alter the capacity of *Pantoea ananatis* AMG501 to form biofilm (Pérez-Montaño et al., 2013). The authors propose that these plants could perceive these bacteria as beneficial. Contrarily, bean seed extract fractions containing AHL-mimic quorum-sensing signals would repress the colonization by AMG501 through the reduction of their ability to form biofilm, thus being recognized as not beneficial (Pérez-Montaño et al., 2013).

Plant roots also secrete compounds that mimic bacterial signals and interfere with quorum sensing regulation in bacteria (Gao et al., 2003). We speculated that differences in biofilm formation caused by rapeseed root exudates could be due to the activity of these compounds. To confirm this hypothesis, the production of quorum sensing signals by isolates in the presence of exudates was evaluated. Since Gram-positive

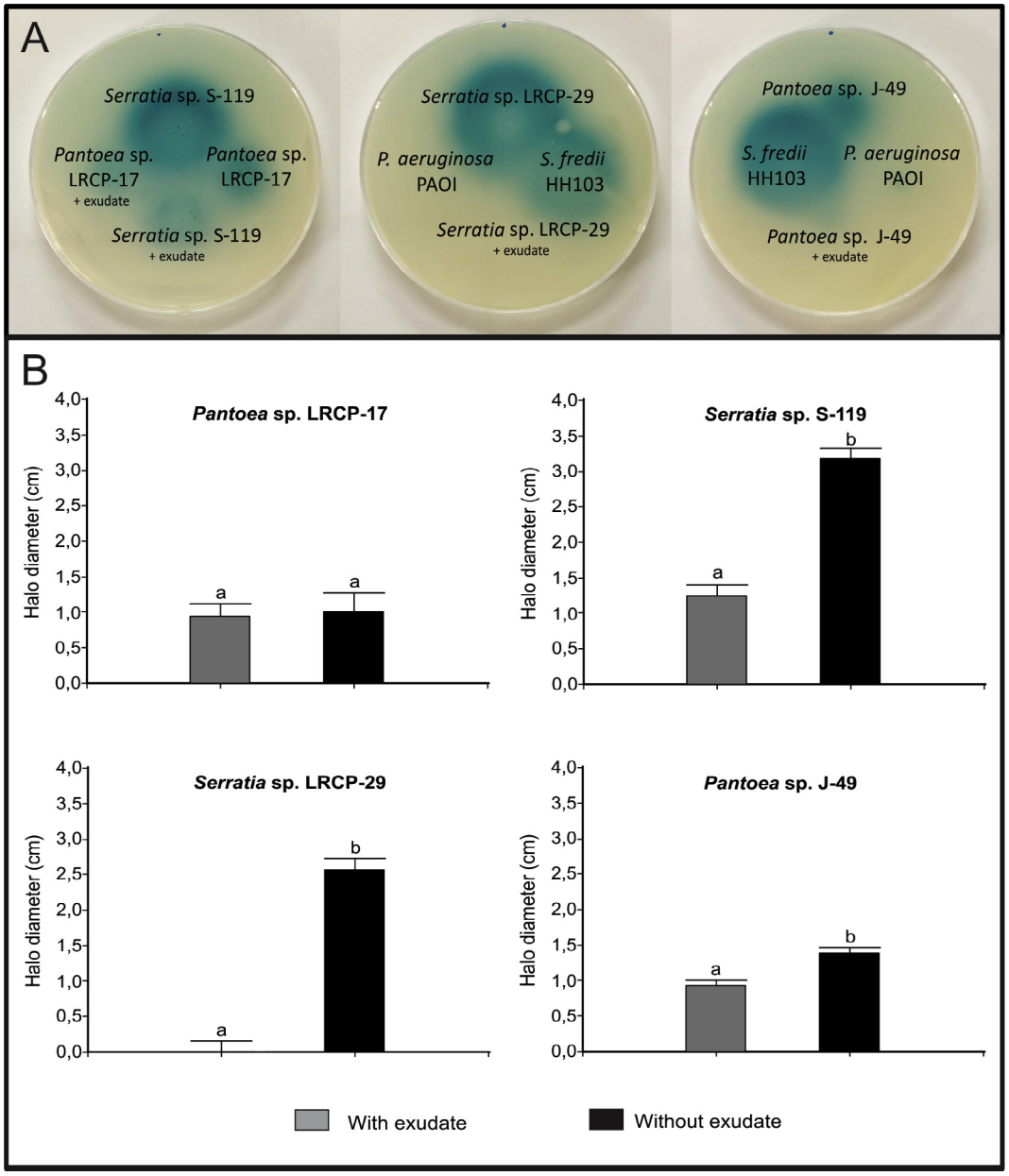

Fig. 4. Effect of rapeseed root exudates on *quorum sensing* signals production. A) Bioassay for detection of AHL-like molecules with long acyl chains using *A. tumefaciens* NTL4 (pZLR4) as biosensor strain. B) Halo diameters. Data represent the mean ± SE of two independent replicates with three repetitions each. Values followed by the same letters are not significantly different ($P \leq 0.05$) for each parameter evaluated.

bacteria do not produce AHL like molecules, the production of these molecules was evaluated only in the Gram-negative bacteria isolatedfrom rapeseed (*Pantoea* sp. LRCP-17, *Serratia* sp. LRCP-29), and from peanut (*Serratia* sp. S119 and *Pantoea* sp. J49).

With the exception of *S. fredii* HH103 (positive control) none of the tested isolates showed positive results for the *C. violaceum* CV026 bioassay, indicating that they were unable to synthesize AHL-like molecules with short acyl chains (Fig. 4). On the other hand, results from the *A. tumefaciens* NTL4 (pZLR4) bioassay revealed that all the isolates produced AHL-like molecules with long acyl chains since presence of a blue halo around the colony resulting from hydrolysis of X-Gal was detected (Fig. 4). Root exudates seem to reduce the *Pantoea* sp. J-49 and *Serratia* sp. S-119 AHL production, as halos with smaller diameters were formed. Instead of that, *Pantoea* sp. LRCP-17 AHL production was not affected by root exudates while *Serratia* sp. LRCP-29 did not produced detectable AHL under this condition, as no halo was formed (Fig. 4).

A link between microbial biofilm formation and quorum sensing has been proposed (Lazar, 2011). However, in this study no relationship between the quantity of AHL produced and the ability of the strains to form biofilm was found. For example, even when the *Serratia* sp. S119 and *Pantoea* sp. J49 AHL production was decreased by root exudates, their biofilm formation abilities were stimulated. In *Pantoea* sp. LRCP-17 AHL production was not affected by root exudates but its ability to form biofilm was stimulated. *Serratia* sp. LRCP-29 short chain AHL production was prevented in the presence of exudates but its ability to form biofilm was modified (Table 3, Fig. 4). In summary, from these results we concluded that under the conditions used in this study, rapeseed root exudates affect the AHL production of most of the isolates tested. However, this effect does not always results in inhibition of biofilm formation capacity. Therefore, and in line with those reported by Perez Montaño et al. (2013), one may speculate that root exudates from rapeseed plants induce or inhibit quorum sensing systems depending on the associated bacterial strain. Other possibility is that the quantity of AHL produced are more related with the architecture of

biofilm formed than with the ability to form biofilm, as it was reported for *Pseudomonas aeruginosa*. On the other hand, it has been proposed that novel alternative signaling molecules, in addition to AHL, could be used to regulate bacterial biofilm formation (Deep et al., 2011).

3.5. Effects of PSB inoculation in field assay

Most studies conducted to evaluate the effect of PSB inoculation were developed under controlled conditions. Often, when bacteria showing the best performance in these studies are evaluated under field conditions, benefits from their inoculation are not detected (Riggs et al., 2001; Gyaneshwar et al., 2001). Some of the factors that can affect the behavior of bacteria in soil are nutrient content (Muthukumarasamy et al., 1999), soil type (de Oliveira et al., 2006) and the host plant variety (Yanni et al., 1997; Muñoz-Rojas and Caballero-Mellado, 2003; de Oliveira et al., 2006). Diverse PSB able to promote the growth of chickpea, wheat, corn, sugarcane and barley crops among others were evaluated in field assays (Pal, 1998; Singh and Kapoor, 1999; Sundara et al., 2002; Zaidi et al., 2003; Babana and Atoun, 2006; Zaidi and Khan, 2006; Mehrvarz et al., 2008; Yazdani et al., 2009). However, few studies have been conducted to evaluate PSB inoculation in rapeseed. In this work, phosphate solubilizing isolates, able to promote the rapeseed growth in chamber trials (*Bacillus* sp. LRCP-4, *Bacillus* sp. LRCP-3 *Bacillus* sp. LRCP-2, *Bacillus* sp. LTAD-52, *Pantoea* sp. LRCP-17, *Serratia* sp. LRCP-29 and *Arthrobacter* sp. LRCP-11) were evaluated in field assay.

At the rosette developmental stage (3 months after planting) no significant differences in the fresh and dry weights of inoculated plants, fertilized and negative controls were observed, except in plants inoculated with *Bacillus* sp LRCP-4 that showed an increase in the air fresh weight, compared with fertilized plants (Table 4). We speculate that, at this stage, plant P requirements are provided by soil. Therefore, the increases observed in the dry or fresh plant weight at this stage could not be attributed to phosphate solubilizing activity of the inoculated strains.

In contrast, at the flowering developmental stage, plants inoculated with *Bacillus* sp. LRCP-4, *Pantoea* sp. LRCP-17, *Arthrobacter* sp. LRCP-11 and *Bacillus* sp. LTAD-52 showed a significant increase in their air fresh weights (Table 4). Air dry weight was also increased by 55% in plants inoculated with *Bacillus* sp. LRCP-4 compared to fertilized plants. The fact that fertilized (positive control) and unfertilized (negative control) plants show similar dry weights may be due to plant P requirements are covered with the soil available level at this developmental stage.

At ripening developmental stage, as it was expected, a response to P fertilization was observed, and the contribution of PSB inoculation was more evident. Shoot dry weight of plants inoculated with *Bacillus* sp. LRCP-2 and *Arthrobacter* sp. LRCP-11 reached values similar to fertilized plants, while those from plants inoculated with *Bacillus* sp. LTAD-52, *Bacillus* sp. LRCP-4, *Pantoea* sp. LRCP-17 and *Serratia* sp. LRCP-29 exceeded by 51% the values reached by fertilized plants (Table 4), suggesting the action of another growth promoter mechanism in addition to phosphate solubilization.

Table 5
Effect of PBS inoculation on yield parameters.

Treatments	Yield Kg ha^{-1}	Weigth 1000 seed Kg	H.I.[†]
Fertilized uninoculated	2726bc	3.13b	0.34a
Negative control	2188a	2.68a	0.35ab
Bacillus sp. LTAD-52	2137a	2.58a	0.034a
Bacillus sp. LRCP-2	2705bc	2.66a	0.38abc
Bacillus sp. LRCP-3	2759bc	2.61a	0.38abc
Bacillus sp. LRCP-4	2951cd	2.69a	0.36abc
Serratia sp. LRCP-29	3154d	2.69a	0.41c
Arthrobacter sp. LRCP-11	2633b	2.72a	0.40bc
Pantoea sp. LRCP-17	2652b	2.63a	0.34a

[†] **H.I.**: Harvest index. Values within a column followed by the same letter are not significantly different at P ≤ 0.05.

Seed yield from inoculated plants were similar or higher than those from fertilized plant (except in plants inoculated with *Bacillus* sp. LTAD-52). The weight of 1000 seeds from inoculated plants was similar to the negative control but lower than values from fertilized plants (Table 5). From these results it is clear that, with the exception of *Bacillus* sp. LTAD-52, inoculation with the PSB tested promotes a significant increase in the number of grain produced per ha. The ratio of yield to above-ground biomass is termed the Harvest Index (Fageria, 1992). The high harvest index determined in plants inoculated with *Serratia* sp. LRCP-29 compared to fertilized plants (Table 5), indicates an increase in the partitioning efficiency of assimilates to the seed, which is also evidenced by the higher seed yield of inoculated compared to fertilized plants.

Kloepper et al. (1988) reported that seed yield increases from 6 to 13% in rapeseed plants inoculated with PSB isolated from the rhizosphere of this crop. In accordance with this work, Madani et al. (2011) reported an increase in yield from rapeseed co-inoculation with the PSB *Pantoea agglomerans* and *Pseudomonas putida* P13 P5. Similar results were reported by Mohammadi and Rokhzadi (2012) in plants co-inoculated with *Pseudomonas putida* and *Bacillus lentus*. On the other hand, Dehpouri et al. (2015) found that simultaneous soil fertilization with 50 kg P ha^{-1} and plant inoculation with the PSB *Pseudomonas putida*, *Pseudomonas fluorescens* increased the seed yield.

4. Conclusions

Application of phosphate solubilizing bacteria to promote plant growth in agriculture is increasing steadily due to recent advances in the understanding of the microorganism-plant interaction. There are a large number of commercial products containing PGPR for different crops. However, rapeseed has been poorly studied in this aspect, even

Table 4
Effect of PBS inoculation on shoot fresh weigh (SFW), shoot dry weight (SDW) and phosphorus content (P) in the rosette, flowering and ripening stages.

Treatments	Rossete			Flowering		Ripening
	SFW g m$^{2-1}$	SDW g m$^{2-1}$	P mg planta^{-1}	SFW g m$^{2-1}$	SDW g m$^{2-1}$	SDW g m$^{2-1}$
Fertilized uninoculated	394.57ab	394.02ab	4.63a	5832a	1393ab	1337b
Negative control	412.56bc	412.56bc	5.24a	5663a	1453ab	969a
Bacillus sp. LTAD-52	394.02ab	394.02ab	4.84a	8897d	1229ab	2006d
Bacillus sp. LRCP-2	417.02bc	417.02bc	3.43a	6495ab	1303ab	1329b
Bacillus sp. LRCP-3	478.95c	478.95c	4.27a	5562a	1183ab	1212ab
Bacillus sp. LRCP-4	631.74d	631.74d	3.63a	11864e	2161c	2031d
Serratia sp. LRCP-29	443.86bc	443.86bc	4.35a	5919ab	1102a	1589c
Arthrobacter sp. LRCP-11	338.21a	338.21a	4.30a	6882bc	1364ab	1391bc
Pantoea sp. LRCP-17	468.03bc	468.03bc	3.76a	7711c	1685bc	1891d

Values within a column followed by the same letter are not significantly different at P ≤ 0.05.

though it is the second most important oilseed crop in the world after soybeans. The results presented in this work extend the knowledge of the diversity of bacteria associated with rapeseed plant, describing for the first time that strains from the genera *Bacillus*, *Serratia*, *Arthrobacter* and *Pantoea*are able to increase significantly their growth. Root exudates play an important role in the microorganism-plant interactions. We found that compounds exuded by the rapeseed roots do not affect the growth and survival of the isolates tested, induced the biofilm formation and affected the *quorum sensing* signals production of some of them. However, this does not seem to affect the efficiency to enhance plant growth of the studied isolates. Field inoculation with phosphate solubilizing bacteria, not only increased significantly plant growth, but also the crop yield (from 21 to 40%), reaching values similar to or even higher than the fertilized control. In summary, this research is a contribution to the development of a biotechnological strategy such as inoculation with phosphate solubilizing bacteria, which enables rapeseed growth promotion and crop yield increase, minimizing the use of chemical fertilizers and contributing to the development of sustainable agriculture.

References

Ahmed, H.F.S., El-araby, M.M.I., 2012. Evaluation of the influence of nitrogen fixing, phosphate solubilizing and potash mobilizing biofertilizers on growth, yield, and fatty acid constituents of oil in peanut and sunflower. Afr. J. Biotechnol. 11, 10079–10088.

Alexander, M., 1980. Transformaciones microbianas del fósforo. In: Introducción a la microbiología del suelo. AGT editor, México, pp. 355–371.

Altschul, S., Madden, T., Schäffer, A., Zhang, J., Zhang, Z., Miller, W., Lipman, D., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search. Nucl. Acids Res. 25, 3389–3402.

Annous, B.A., Solomon, E.B., Cooke, P.H., Burke, A., 2005. Biofilm formation by Salmonella spp. on cantaloupe melons. J. Food Safe 25, 276–287.

Babana, A., Atoun, H., 2006. Biological system for improving the availability of Tilemsi phosphate rock for wheat (*Triticum aestivum* L.) cultivated in Mali. Plant Soil 287, 51–58.

Badri, D.V., Vivanco, J.M., 2009. Regulation and function of root exudates. Plant Cell Environ. 32, 666–681.

Bais, H.P., Fall, J.M., Vivanco, R., 2004. Biocontrol of *Bacillus subtilis* against infection of arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol. 134, 307–319.

Bais, H.P., Weir, L.P., Perry, L.G., Gilroy, S., Vivanco, J.M., 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annu. Rev. Plant Biol. 57, 233–266.

Bertrand, H., Nalin, R., Bally, R., Cleyet-Marel, J., 2001. Isolation and identification of the most efficient plant growth-promoting bacteria associated with canola (*Brassica napus*). Biol. Fertil. Soils 33, 152–156.

Brader, G., Compant, S., Mitter, B., Trognitz, F., Sessitsch, A., 2014. Metabolic potential of endophytic bacteria. Curr. Opin. Biotechnol. 27, 30–37.

Brameyer, S., Bode, H.B., Heermann, R., 2015. Languages and dialects: bacterial communication beyond homoserine lactones. Trends Microbiol. 23, 521–523.

Buendía-Clavería, A.M., Chamber, M., Ruiz-Sainz, J.E., 1989. A comparative study of the physiological characteristics, plasmid content and symbiotic properties of different *Rhizobium fredii* in European soils. Syst. Appl. Bacteriol. 17, 155–160.

Cha, C., Gao, P., Chen, Y., Shaw, P.D., Farrand, S.K., 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Mol. Plant Microbe Interact. 11, 1119–1129.

Chabot, R., Anton, H., Cescas, M.C., 1996. Growth promotion of maize and lettuce by phosphate-solubilizing *Rhizobium leguminosarum* biovar phaseoli. Plant Soil 184, 311–321.

Costerton, J., Geesey, G.G., Chen, K.J., 1978. How bacteria stick. Sci. Am. 238, 86–95.

Danhorn, T., Fuqua, C., 2007. Biofilm formation by plant-associated bacteria. Annu. Rev. Microbiol. 61, 401–422.

de Freitas, J., Banerjee, M., Germida, J., 1997. Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). Biol. Fertil. Soils 24, 358–364.

de Oliveira, A.L.M., de Canuto, E.L., Urquiaga, V., Reis, M., Baldani, J.I., 2006. Yield of micropropagated sugarcane varieties in different soil types following inoculation with diazotrophic bacteria. Plant Soil 284, 23–32.

de Weert, S., Vermeiren, H., Mulders, I.H.M., Kuiper, I., Hendrickx, N., Bloemberg, G.V., Vanderleyden, J., 2002. Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. Mol. Plant Microbe Interact. 15, 1173–1180.

Deep, A., Chaudhary, U., Gupta, V., 2011. Quorum sensing and bacterial pathogenicity: from molecules to disease. J. Lab. Physicians 3, 4.

Dehpouri, F., Vahedi, A., Yasari, E., Chepi, O.G., Haddadi, M.H., 2015. Effects of phosphate solubilizing bacteria and mineral phosphorous levels on yield and yield components of canola Hyola 401 cultivar. Agric. Adv. 4, 7–14.

Dennis, P.G., Miller, A.J., Hirsch, P.R., 2010. Are root exudates more importante sources of rhizodeposits in structuring rhizosphere bacterial communities? FEMS Microbiol. Ecol. 72, 313–327.

Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M., Robledo, Y.C., 2014. InfoStat versión 2014. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina.

Eberhard, A., Burlingame, A.L., Eberhard, C., Kenyon, G.L., Nealson, K.H., Oppenheimer, N.J., 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry 20, 2444–2449.

Fageria, N.K., 1992. Biological yield, economic yield and harvest index. In: Fageria, N.K. (Ed.), Maximizing Crop Yields. Marcel Dekker, New York, pp. 10–13.

Farrand, S.K., Qin, Y., Oger, P., 2002. Quorum-sensing system of Agrobacterium plasmids: analysis and utility. Methods Enzymol. 358, 452–484.

Gao, M.S., Teplitski, M., Robinson, J.B., 2003. Production of substances by *Medicago truncatula* that affect bacterial quorum sensing. Mol. Plant Microbe Interact. 16, 827–834.

Gyaneshwar, P., James, E.K., Mathan, N., Reddy, P.M., Reinhold-Hurek, B., Ladha, J.K., 2001. Endophytic colonization of rice by a diazotrophic strain of *Serratia marcescens*. J. Bacteriol. 183, 2634–2645.

Hall, J.A., Peirson, D., Ghosh, S., Glick, B.R., 1996. Root elongation in various agronomic crops by the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. Isr. J. Plant Sci. 44, 37–42.

Hameeda, B., Harini, G., Rupela, O.P., Wani, S.P., Reddy, G., 2008. Growth promotion of maize by phosphate-solubilizing bacteria isolated from composts and macrofauna. Microbiol. Res. 163, 234–242.

Hamon, M.A., Lazazzera, B.A., 2001. The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. Mol. Microbiol. 42, 1199–1209.

Hartmann, A., Schmid, M., van Tuinen, D., Berg, G., 2009. Plant driven selection of microbes. Plant Soil 321, 235–257.

Hinsinger, P., Bengough, A.G., Vettrlein, D.I., Young, M., 2009. Rhizosphere: biophysics, biogeochemistry and ecological relevance. Plant Soil 321, 117–152.

Hoagland, D.R., Arnon, D.I., 1950. Water culture method for growing plants without soil. Agricultural experiment Station Circular 347, California.

Holloway, B.W., 1955. Genetic recombination in *Pseudomonas aeruginosa*. J. Gen. Microbiol. 13, 572–581.

Ibáñez, F., Angelini, J., Taurian, T., Tonelli, M.L., Fabra, A., 2009. Endophytic occupation of peanut root nodules by opportunistic Gammaproteobacteria. Syst. Appl. Microbiol. 32, 49–55.

Iriarte, L., Valetti, O., 2008. En "Cultivo de Colza" 1ª edición. C.A. Bs. As. INTA.

Karthikeyan, M., Bhaskaran, R., Radhika, K., Mathiyazhagan, S., Jayakumar, V., Sandosskumar, R., Velazhahan, R., 2005. Endophytic *Pseudomonas fluorescens* Endo2 and Endo35 induce resistance in black gram (*Vigna mungo* L. Hepper) to the pathogen *Macrophomina phaseolina*. J. Plant Interact. 1, 135–143.

Kloepper, J.W., Lifshitz, R., Zablotowicz, R.M., 1989. Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol. 7, 39–49.

Kloepper, J.W., Hume, D.J., Scher, F.M., Singleton, C., Tipping, B., Laliberté, M., Frauley, K., Kutchaw, T., Simonson, C., Lifshitz, R., Zaleska, I., Lee, L., 1988. Plant growth-promoting rhizobacteria on canola (rapeseed). Plant Dis. 72, 42–46.

Kuklinsky-Sobral, J., Araújo, W., Mendes, R., Geraldi, I., Pizzirani-Kleiner, A., Azevedo, J., 2004. Isolation and characterization of soybean associated bacteria and their potential for plant growth promotion. Environ. Microbiol. 6, 1244–1251.

Kumar, R., Bhatia, R., Kukreja, K., Behl, R.K., Dudeja, S.S., Narula, N., 2007. Establishment of Azotobacter on plant roots: chemotactic response, development and analysis of root exudates of cotton (*Gossypium hirsutum* L.) and wheat (*Triticum aestivum* L.). J. Basic Microbiol. 47, 436–439.

Lazar, V., 2011. Quorum sensing in biofilms how to destroy the bacterial citadels or their cohesion/power? Anaerobe 17, 280–285.

Lugtenberg, B.J.J., Kravchenko, L.V., Simons, M., 1999. Tomato seed and root exudate sugars: composition, utilization by *Pseudomonas* biocontrol strains and role in rhizosphere colonization. Environ. Microbiol. 1, 439–446.

Madani, H., Malboobi, M.A., Stoklosa, A., 2011. Biological and chemical phosphorus fertilizers effect on yield and P accumulation in rapeseed (*Brassica napus* L.). Not. Bot. Horti. Agrobo. 40, 210–214.

Mc Clean, K.H., Wilson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., Daykin, M., Lamb, J.H., Swift, S., Bycroft, B.W., Stewart, G.S.A., Williams, P., 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acyl-homoserine lactones. Microbiology 143, 3003–3011.

Mehrvarz, S., Chaichi, M.R., Alikhani, H.A., 2008. Effects of phosphate solubilizing microorganisms and phosphorus chemical fertilizer on yield and yield components of Barely (*Hordeum vulgare* L.). Am.-Euras. J. Agric. Environ. Sci. 3, 822–828.

Mehta, S., Nautiyal, C.S., 2001. An efficient method for qualitative screening of phosphate-solubilizing bacteria. Curr. Microbiol. 43, 51–56.

Mendez-Castro, F.A., Alexander, M., 1983. Method for establishing a bacterial inoculum on corn roots. Appl. Environ. Microbiol. 45, 248–254.

Miller, J.H., 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mohammadi, K., Rokhzadi, A., 2012. An integrated fertilization system of canola (*Brassica napus* L.) production under different crop rotations. Ind. Crops Prod. 37, 264–269.

Molina, M.A., Ramos, J.L., Espinosa-Urgel, M., 2003. Plant-associated biofilms. Rev. Environ. Sci. Biotechnol. 2, 99–108.

Muñoz-Rojas, J., Caballero-Mellado, J., 2003. Population dynamics of *Gluconacetobacter diazotrophicus* in sugarcane cultivars and its effect on plant growth. Microb. Ecol. 46454–46464.

Murphy, J., Riley, J.P., 1962. A modified single solution method for the determination of phosphore in natural waters. Anal. Chim. Acta 27, 31–36.

Muthukumarasamy, R., Revathí, G., Lakshminarasimhan, C., 1999. Diazotrophic

associations in sugarcane cultivation in South India. Trop. Agric. 76, 171–178.
Ng, W.L., Bassler, B.L., 2009. Bacterial quorum-sensing network architectures. Annu. Rev. Genet. 43, 197–222.
O'Toole, G.A., Kolter, R., 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. 28, 449–461.
Pal, K.K., Tilak, K.V.B.R., Saxena, A.K., Dey, R., Singh, C.S., 2001. Suppression of maize root diseases caused by *Macrophomina phaseolina*, *Fusarium moniliforme* and *Fusarium graminearum* by plant growth promoting rhizobacteria. Microbiol. Res. 156, 209–223.
Pal, S., 1998. Interactions of an acid tolerant strain of phosphate solubilizing bacteria with a few acid tolerant crops. Plant Soil 198, 169–177.
Pavan, M.A., Bloch, M.F., Zempulski, H.C., Miyazawa, M., Zocoler, D.C., 1992. Manual de análise química do solo e controlede qualidade. Instituto Agronômico do Paraná, Brasil, pp. 46.
Peix, A., Rivas-Boyero, A.A., Mateos, P.F., Rodríguez-Barrueco, C., Martínez-Molina, E., Velázquez, E., 2001. Growth promotion of chickpea and barley by a phosphate solubilizing strain of *Mesorhizobium mediterraneum* under growth chamber conditions. Soil Biol. Biochem. 33, 103–110.
Pérez-Montaño, F., Jiménez-Guerrero, I., Contreras Sánchez-Matamoros, R., López-Baena, F.J., Ollero, F.J., Rodríguez-Carvajal, M.A., Bellogín, R.A., 2013. Rice and bean AHL-mimic quorum-sensing signals specifically interfere with the capacity to form biofilms by plant-associated bacteria. Res. Microbiol. 164, 749–760.
Ramey, B.E., Koutsoudis, M., Von Bodman, S.B., Fuqua, C., 2004. Biofilm formation in plant-microbe associations. Curr. Opin. Microbial. 7, 602–609.
Renwick, A.R., Campbell, A., Coe, S., 1991. Assessment of in vivo screening systems for potential biocontrol agents of *Gaeumannomyces graminis*. Plant Physiol. 40, 524–532.
Riggs, P.J., Chelius, M.K., Iniguez, A.L., Kaeppler, S.M., Triplett, E.W., 2001. Enhanced maize productivity by inoculation with diazotrophic bacteria. Aust. J. Plant Physiol. 28, 829–836.
Romero, D., de Vicente, A., Rakotoaly, R.H., Dufour, S.E., Veening, J.W., Arrebola, E., Cazorla, F.M., Kuipers, O.P., Paquot, M., Perez-Garcia, A., 2007. The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Mol. Plant Microbe Interact. 20, 430–440.
Salimpour, S., Khavazi, K., Nadian, H., Besharati, H., Miransari, M., 2010. Enhancing phosphorous availability to canola (*Brassica napus* L.) using P solubilizing and sulfur oxidizing bacteria. Aust. J. Crop Sci. 4, 330–334.
Seneviratne, G., Jayasinghearachchi, H.S., 2005. A rhizobial biofilm with nitrogenase activity alters nutrient availability in a soil. Soil Biol. Biochem. 37, 1975–1978.
Shi, Y., Wang, Y., Zhou, Q., Liu, H., Li, B., Luo, J., Xie, G., 2012. Effect of rice root exudates and strain combination on biofilm formation of *Paenibacillus polymyxa* and *Paenibacillus macerans*. Afr. J. Microbiol. Res. 6, 3343–3347.
Singh, S., Kapoor, K.K., 1999. Inoculation with phosphate-solubilizing microorganisms and a vesicular-arbuscular mycorrhizal fungus improves dry matter yield and nutrient uptake by wheat grown in a sandy soil. Biol. Fertil. Soils 28, 139–144.
Somasegaran, P., Hoben, H.J., 1994. Handbook for Rhizobia: Methods in Legume-rhizobium Technology. Springer-Verlag Inc., New York, pp. 450.
Somers, E., Vanderleyden, J., Srinivasan, M., 2004. Rhizosphere bacterial signalling: a love parade beneath our feet. Crit. Rev. Microbiol. 304, 205–240.
Sood, S.G., 2003. Chemotactic response of plant-growth-promoting bacteria towards roots of vesicular-arbuscular mycorrhizal tomato plants. FEMS Microbiol. Ecol. 45, 219–227.
Subba Rao, N.S., 1988. Biofertilizers in agriculture. M. Dehli. Vance, pp. 16–76.
Sundara, B., Natarajan, V., Hari, K., 2002. Influence of phosphorus solubilizing bacteria on the changes in soil available phosphorus and sugarcane yields. Field Crops Res. 77, 43–49.
Taheri, E., Soleymani, A., Javanmard, H.R., 2012. The effect of different Nitrogen Levels on oil yield and harvest index of two spring rapeseed cultivars in Isfahan region. Int. J. Agric. Crop Sci. 4, 1496–1498.
Tan, S., Yang, C., Mei, X., Shen, S., Raza, W., Shen, Q., Xu, Y., 2013. The effect of organic acids from tomato root exudates on rhizosphere colonization of *Bacillus amyloliquefaciens* T-5. Appl. Soil Ecol. 64, 15–22.
Taurian, T., Anzuay, M.S., Angelini, J.G., Tonelli, M.L., Ludueña, L., Pena, D., Ibáñez, F., Fabra, A., 2010. Phosphate-solubilizing peanut associated bacteria: screening for plant growth-promoting activities. Plant Soil 329, 421–431.
Taurian, T., Morón, B., Soria-Díaz, M.E., Angelini, J.G., Tejero-Mateo, P., Gil-Serrano, A., Fabra, A., 2008. Signal molecules in the peanut-bradyrhizobia interaction. Arch. Microbiol. 189, 345–356.
Teplistki, M., Robinson, J.B., Bauer, W.D., 2000. Plants secrete substances that mimic bacterial N-acyl homoserine lactone signal activities and affect population density dependent behaviors in associated bacteria. Mol. Plant Microbe Int. 13, 637–648.
Vessey, J.K., 2003. Plant growth promoting rhizobacteria as biofertilizer. Plant Soil 255, 571–586.
Vincent, J.M., 1970. A Manual for the Practical Study of Root Nodule Bacteria. Blackwell Scientific, Oxford.
Wakelin, S.A., Warren, R.A., Harvey, P.R., Ryder, M.H., 2004. Phosphate solubilization by *Penicillium* spp. closely associated with wheat roots. Biol. Fert. Soils 40, 36–43.
Walker, T.S., Bais, H.P., Déziel, E., Schweizer, H.P., Rahme, L.G., Fall, R., Vivanco, J.M., 2004. *Pseudomonas aeruginosa*-plant root interactions. Pathogenicity, biofilm formation, and root exudation. Plant Physiol. 134, 320–331.
Walker, T.S., Bais, H.P., Grotewold, E., Vivanco, J.M., 2003. Root exudation and rhizosphere biology. Plant Physiol. 132, 44–51.
Waters, C.M., Lu, W., Rabinowitz, J.D., Bassler, B.L., 2008. Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of vpsT. J. Bacteriol. 190, 2527–2536.
Webb, J.S., Givskov, M., Kjelleberg, S., 2003. Bacterial biofilms: prokaryotic adventures in multicellularity. Curr. Opin. Microbiol. 6, 578–585.
Winson, M.K., Camara, M., Latifi, A., Foglino, M., Chhabra, S.R., Daykin, M., Bally, M., Chapon, V., Salmond, G.P.C., Bycroft, B.W., Lazdunski, A., Stewart, G.S.A.B., Williams, P., 1995. Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U.S.A. 92, 9427–9943.
Yanni, Y.G., Rizk, R.Y., Corich, V., Squartini, A., Ninke, K., Philip-Hollingsworth, S., Orgambide, G., de Bruijn, F.D., Stoltzfus, J., Buckley, D., Schmidt, T.M., Mateos, P.F., Ladha, J.K., Dazzo, F.B., 1997. Natural endophytic associations between *Rhizobium leguminosarum bv. trifolii* and rice roots and assessment of its potential to promote rice growth. Plant Soil 194, 99–114.
Yazdani, M., Bahmanyar, M.A., Pirdashti, H., Esmaili, M.A., 2009. Effect of phosphate solubilisation microorganisms (PSM) and plant growth promoting rhizobacteria (PGPR) on yield and yield components of Corn (*Zea mays* L.). Proc. World Acad. Sci. Eng. Technol. 3790–3792.
Yuan, J., Zhang, N., Huang, Q., Raza, W., Li, R., Vivanco, J.M., Shen, Q., 2015. Organic acids from root exudates of banana help root colonization of PGPR strain *Bacillus amyloliquefaciens* NJN-6. Sci. Rep 5pp. 13438.
Zaidi, A., Khan, M.S., 2006. Co-inoculation effects of phosphate solubilizing microorganisms and *Glomus fasciculatumon* on green gram-*Bradyrhizobium* symbiosis. Turk. J. Agric. 30, 223–230.
Zaidi, A., Khan, M.S., Amil, M., 2003. Interactive effect of rhizotrophic microorganisms on yield and nutrient uptake of chickpea (*Cicer arietinum* L.). Eur. J. Agron. 19, 15–21.
Zheng, X.Y., Sinclair, J.B., 1996. Chemotactic response of *Bacillus megaterium* strain B153-2-2 to soybean root and seed exudates. Physiol. Mol. Plant Path. 48, 21–35.