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Lettuce and rhizosphere microbiome responses to growth promoting *Pseudomonas* species under field conditions

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Abstract

Plant growth promoting rhizobacteria (PGPR) are well described and recommended for several crops worldwide. However, one of the most common problems in PGPR research is the difficulty in obtaining reproducible results. Furthermore, few studies have evaluated plant growth promotion and soil microbial community composition due to bacterial inoculation under field conditions. Here we evaluated the effect of 54 *Pseudomonas* strains on lettuce (*Lactuca sativa*) growth. The 12 most promising strains were phylogenetically and physiologically characterized for plant growth-promoting traits including phosphate solubilization, hormone production and antagonism to pathogen compounds and their effect on plant growth under farm field conditions. Additionally, the impact of beneficial strains on the rhizospheric bacterial community was evaluated for inoculated plants. The strains IAC-RBcr4 and IAC-RBru1, with different plant growth promoting traits, improved lettuce plant biomass yields up to 30%. These two strains also impacted rhizosphere bacterial groups including *Isosphaera* and *Pirellula* (phylum Planctomycetes) and *Acidothermus, Pseudolabrys* and *Singusphaera* (phylum Actinobacteria). This is the first study to demonstrate consistent results for the effects of *Pseudomonas* strains on lettuce growth promotion for seedlings and plants grown under tropical field conditions.

Introduction

The use of fungi and bacteria as inoculants for enhancing crop production is a viable alternative to traditional fertilizers for purposes of enhancing plant productivity and improving soil quality without causing environmental pollution (Berg 2009; Bhardwaj *et al*. 2014). Farmers worldwide have begun using plant growth promoting microorganisms to support sustainable food production (Vessey 2003). Among the

beneficial microorganisms, bacterial groups known as plant growth promoting rhizobacteria (PGPR) are capable of colonizing plant rhizospheres (Kloepper and Schroth 1978).

Rhizobacteria have been shown to improve plant growth even under adverse conditions such as chemically contaminated soil or hydric stress, and also can act as antagonists of plant pathogens (Vargas *et al*. 2014; Dutta *et al*. 2014; Santiago *et al*. 2015; Lampis *et al*. 2015). Plant growth promotion has been shown to occur through various mechanisms involving several rhizobacterial metabolites. Some metabolites trigger solubilization of insoluble phosphates and production of auxin-type indole-3-acetic acid (IAA), which improves root growth (Seergeva *et al*. 2007; Hariprasad and Niranjana 2009; Oteino *et al*. 2015). In contrast, hydrogen cyanide is an example of a rhizobacterial metabolite that can inhibit plant pathogens. Furthermore, through biological nitrogen fixation, rhizobacterial symbionts provide essential nutrients to many plant species (Frapolli *et al*. 2012; Rodríguez-Blanco *et al*. 2015).

Physicochemical soil conditions and the endogenous soil microbial community also contribute to the overall efficacy of plant-rhizospheric bacteria interaction. Plant growth promotion by PGPR is dependent on agricultural management and soil type (Berger *et al*. 2013; Schreiter *et al*. 2014a). The use of rhizobacteria as inoculants has previously resulted in inconsistent plant responses and few studies have evaluated specific plant-microorganism interactions under field conditions. To obtain more consistent results, some studies have evaluated the effect of inoculation with multiple bacterial strains from either the same or different taxonomic groups (Shanmugam & Kanoujia 2011; Dutta *et al*. 2014; Ibañez *et al*. 2014). For soybean plants, the co-inoculation of endophytes and PGPR, including *Pseudomonas* strains, benefited plant growth and yield by increasing nitrogen fixation (Prakamhang *et al*. 2015). However, for some cases studied, combinations of PGPRs did not show benefits. When *Pseudomonas* and *Azospirillum* species were co-inoculated, the *Pseudomonas* species inhibited colonization by *Azospirillum* and plant growth

promotion was not achieved (Couillerot *et al*. 2011). Depending on the *Pseudomonas* strain, co-inoculation is often not recommended because of antagonistic activity with respect to other beneficial bacteria (Pierson & Weller 1994). However, results of other studies suggest that combinations of pseudomonads can produce synergistic effects without competition between strains, thus benefiting the host plant (Jamali *et al*. 2009; Ibañez *et al*. 2014).

In lettuce (*Lactuca sativa* L.) crop research, several studies have described PGPR effects on plant growth in a healthy and sustainable manner (Grosch *et al*. 2012; Schreiter *et al*. 2014a, b; Kröber *et al*. 2014). Lettuce is the most consumed vegetable in Brazil with an estimated annual production of 28,815 tons (Sandri *et al*. 2007; Agrianual 2012; Sala & Costa *et al*. 2012). Despite the importance of lettuce production in Brazil, there are few studies describing the use of rhizobacteria as lettuce growth promoters (Cipriano *et al*. 2013; Pinto *et al*. 2014; Corrêa *et al*. 2015). These studies, all carried out under greenhouse conditions, demonstrated better development and improvement of root and shoot growth with lower pesticide use and lower pathogen frequencies for bacterial inoculated plants compared to the same characteristics of untreated plants. However, there are no studies on the effects of these growth promoting bacteria in tropical field conditions. Furthermore, the effect of rhizobacteria on the microbial community comprising the lettuce rhizosphere is not known. Information about effects on the lettuce rhizosphere microbial community could inform future studies about inoculum recommendations for lettuce cultivation.

The present study aimed to broaden the knowledge about the effect of *Pseudomonas* strains on lettuce plant development, on rhizosphere biological properties (microbial biomass and activity), and on composition of the lettuce rhizosphere microbial community. Our hypothesis was that *Pseudomonas* strains with different physiological traits would benefit plant growth at different stages of development and growth with minimal of no impact on the microbial community in the rhizosphere of field-grown lettuce. In order to test this hypothesis, we characterized

growth promotion-related metabolites for 54 *Pseudomonas* strains and their effects on lettuce seedlings in greenhouse. In addition, we evaluated the performance of lettuce plants inoculated with 12 selected pseudomonads under field conditions and the effects and outcome on rhizosphere microbial community assembly in response to *Pseudomonas* spp. inoculations.

Materials and Methods

Bacterial strain selection

All *Pseudomonas* strains were obtained from the Agronomic Institute of Campinas (IAC, Brazil) culture collection, isolated from healthy lettuce (*Lactuca sativa* L.), chrysanthemum (*Dendranthema grandiflorum*), rucola (*Eruca sativa*) or maize (*Zea mays*) rhizospheres. This study was divided into two parts: (I) screening of 54 strains based on plant growth traits in lettuce seedlings under greenhouse conditions and (II) molecular characterization of 12 strains selected from part I by sequencing 16S rRNA and *nifH* genes, and evaluation of rhizobacteria-lettuce interactions in lettuce seedling and adult plants under field conditions.

Part I

In vitro screening of strains for established plant growth promoting traits

Hydrogen cyanide (HCN) − A total of 54 pseudomonads strains were grown on King's B (KB) (King *et al*. 1954) culture medium with glycine. The production of HCN was assessed according Bakker and Schippers (1987). Moistened filter paper with picric acid solution (5%) and

Na_2CO_3 (2%) was added at the top of the Petri dishes and incubated at 28°C for 36 h. The experiments were performed in triplicate for each strain. The ability to produce HCN was indicated by the paper's color change from yellow to orange-red.

Indole-3-acetic acid (IAA) – The strains were grown in culture medium with L-tryptophan, the precursor of IAA (Bric *et al*. 1991), covered with a nitrocellulose membrane, and incubated at 28°C in the dark for 24 h. The nitrocellulose membranes were immersed in Salkowski's solution and incubated at room temperature for up to three hours. The red-purplish color halo around the colonies indicated IAA production.

Phosphate solubilization (PS) – The strains were grown on culture medium containing inorganic phosphate ($CaHPO_4$) (Katznelson and Bose, 1959). The experiment was performed in triplicate and carried out daily for five days. The ability of the bacteria to solubilize the calcium phosphate was verified by the development of clear halo surrounding the colonies.

Assessing lettuce seedling growth under greenhouse conditions

The 54 strains were grown in KB liquid medium for 16h at 28°C. Cells were collected by centrifugation (13,000 x*g*, 10 min), suspended in sterile 0.01 mol.L^{-1} $MgSO_4 \cdot 7H_2O$ solution and adjusted to a density of 10^8 colony forming units (CFU) mL^{-1}.

The experiment was carried out in greenhouse at the Agronomic Institute of Campinas, Brazil (22°54′20″S, 47°05′34″W). Due to the large number of strains we analyzed and tested, we divided them among 4 different experiments (Table S1). Seeds of curly lettuce (cv. Verônica, Sakata) were germinated in 200-well seedling trays filled with commercial substrate (Tropstrato HA® Hortaliças). The substrate is composed of a mixture of pine three material and peat soil, treated at high temperature for elimination of plant pathogens. The bacterial suspension (4

mL/seedling) was used to inoculate seedling laps at the 2-leaf stage. The seedlings of the control were drenched with 0.01 mol.L^{-1} $MgSO_4.7H_2O$ instead of the bacterial suspension. All seedling trays were watered daily and fertilized with nutrient solution (Furlani *et al*. 1999) at days 7 and 15 after sowing. Seedling harvest was performed 20 days after sowing (3-leaf stage). Plant growth was determined by dry mass of roots and the shoots separately. The experiment was performed using five replicates with five seedlings per replicate. Lettuce dry mass data was analyzed by ANOVA using the Scott-Knott procedure with $p \leq 0.05$ considered as significant using the R package (Team *et al*., 2005).

Part II

According to the *in vitro* PGPR screening and greenhouse experimental results in Part I, 12 strains were chosen for further analysis in part II of this study.

16S rRNA gene and *nifH* analyses of selected rhizobacteria strains

The 12 strains were grown in 5 mL of Nutrient Broth (28°C for 16-18 h at 5,000 x*g*) and the total DNA extraction was according to Pitcher *et al*. (1989). The DNA quality and quantity were verified by gel electrophoresis (0.6% agarose in 1X TAE buffer). The 16S rRNA gene amplification was performed in 25 µL reactions containing 200 ng of genomic DNA, 2.0 U of *Taq* polymerase (Fermentas), 1X Taq buffer (Fermentas), 0.2 mmol dNTP mixture and 0.4 mmol of primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1100r (5′-AGGGTTGGGGTGGTTG-3′) (Lane, 1985). The PCR conditions were: 95 °C/2 min followed by 30 cycles of 94 °C/1 min, 60 °C/1 min and 72 °C/3 min and, one cycle at 72 °C/5 min (final extension) in a thermocycler (Geneamp PCR System 9700; Perkin-Elmer). PCR products were

purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare) and the sequencing was carried out using an Applied Biosystems-Hitachi ABI Prism 3700 DNA Analyzer. The *Pseudomonas* 16S rRNA gene sequences were aligned with 20 sequences obtained from the NCBI database using ClustalX in MEGA version 6 (Tamura *et al*., 2013). The phylogenetic tree was generated using the neighbor-joining algorithm (Saitou & Nei, 1987) with *Stenotrophomonas maltophilia* as the outgroup.

The amplification of *nifH* was carried out in order to verify the nitrogen fixation ability of the strains using the primers PolF (5′-CCSGAYAARGCBGCTGACTC-3′) and PolR (5′-ATSATYATCGCCCCTGCCGA-3′) (Poly *et al*., 2001). The PCR conditions were the same as described by Poly *et al*. (2001). *Pseudomonas* sp. and *Rhizobium leguminosarum* strains with genomically encoded *nifH* were used as positive controls.

Assessing the effects of selected *Pseudomonas* strains on seedlings and plants under field conditions

The effects of the 12 selected *Pseudomonas* strains on lettuce development was determined at the experimental field of the Ballagro company (23°08' S, 46°27' W), Brazil, under field conditions similar to those for commercial production of lettuce. Three experiments were carried out due to the large number of strains and plants to be analyzed. In experiment 1, between October and December 2012, five strains (IAC-RBal1, IAC-RBal2, IAC-RBal3, IAC-RBcr2 and IAC-RBcr5) were tested. In experiment 2, between February and April of 2013, three strains (IAC-RBmi1, IAC-RBcr1 and IAC-RBcr3) and a mixture of the three strains (Mix) were tested. In experiment 3, between March and May of 2013, five strains (IAC-RBcr4, IAC-RBcr6, IAC-RBru1, IAC-RBal4) and a mixture of the four strains were evaluated.

Lettuce seedlings were obtained and grown in substrate as described in Part I. However, the fertilization regimen was different because the plants remained longer in the field. The seedlings were fertilized twice (days 10 and 20 after sowing) with monoammonium phosphate (100 g/100 L^{-1}) and the mineral fertilizer Pick Up Ca, containing calcium carbonate (50 mL/100 L^{-1}). After four weeks, half of the seedlings at the 4-leaf stage were harvested and the effect of the bacterial inoculation on the plant growth promotion was evaluated based on shoot and root dry masses (as described in part I).

During the second stage of the experiment, the remaining seedlings were reinoculated (10^8 CFU ml^{-1}) with the respective strains and after 24 hours, plants were transferred to the field. The field soil type was sandy-clay-loam. The field was divided into beds of 10 m^2 each. Before transferring the seedlings to the beds, solarization, a common practice for lettuce production in Brazil, was carried out by covering the soil with a 100 μm thick transparent PVC plastic film for 45 days to decrease the incidence of weeds and soil-borne pathogens. The seedlings were planted in beds in five rows with intra-row and inter-row distances of 30 cm each (60 plants per bed). Each treatment included ten replicates arranged randomly and each replicate was composed of five plants. Fertilizer was added based on Van Raij *et al*. (2001) soil chemical analysis (Supporting Information Table S2). Fifteen days before planting, nitrogen, phosphate and potassium were applied at a ratio of 04:14:08, respectively, at 500 kg/ha. During cultivation, the beds with plants were fertilized with 10:10:10 (N:P:K) three times: 400 kg/ha at days 10, 20 and 30 and the environmental temperature was recorded (Supporting Information Table S3). After 4 weeks, plants were harvested for the evaluation of root and shoot growth (assessed as dry mass), and soil attached to the plant roots was collected using a sterile soft brush for analysis of rhizospheric soil biological properties (microbial biomass carbon and basal soil respiration) and microbial community compositional analysis

by 16S rRNA gene sequencing. The rhizospheric soil samples were stored at 4 °C before analysis and at -20 °C for DNA extractions. Control treatments for all three experiments were seedlings and plants without *Pseudomononas* inculation.

Rhizospheric soil analyses

Microbial biomass carbon (MBC)

Biomass was determined by a fumigation-extraction method (Vance *et al*., 1987), for which half of the samples was fumigated with chloroform free of ethanol and the other half were held at room temperature. Carbon extraction was with 0.5 mol.L^{-1} K_2SO_4. The soil extracts were digested by potassium dichromate and then quantified by titration with standardized ammonium sulfate solution. Carbon extracted from lysed cells was calculated based on the difference between the surplus volume of dichromate required for fumigated versus non-fumigated soils.

Basal soil respiration (BR)

Microbial activity was determined according to the method described by Alef (1995). The samples were kept in sealed jars with an Erlenmeyer containing 1N NaOH solution. The solution reacted with the CO_2 released by microbial respiration and formed Na_2CO_3 during incubation at 28 °C for three days. The amount of CO_2 released was assessed by titration with 1N HCl with 1 mL of $BaCl_2$ and three drops of phenolphthalein. The results were expressed in g C/CO_2 g^{-1} day^{-1}.

DNA extraction and 16S rRNA gene amplification of rhizosphere microbiome

Three replicates per treatment of rhizospheric soil from the lettuce plants were collected. From each sample, 2 g of soil was used for total DNA extraction using the DNA PowerSoil kit (MoBio laboratories, Inc.). The yield and quality of the total DNA were determined by spectrophotometry (NanoDrop 1000 Thermo scientific, USA). The rhizospheric bacterial community was determined based on 16S rRNA gene sequencing (V4 region) using the forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and the reverse primer 806R (5′-GGACTACVSGGGTATCTAAT-3′). The PCRs were performed in triplicate with the FastStart™ High Fidelity PCR System (Roche Diagnostics Ltd., Burgess Hill, UK) and amplified in a C1000 Touch thermal cycler (Bio-Rad, Hemel Hempstead, UK). The PCR was based in 25 μL reaction containing 1 μL of DNA template, 5 μl FastStart High Fidelity Enzyme Blend, 10x FastStart High Fidelity Buffer with 1.8 mM $MgCl_2$, 0.2 mM of each dNTP (Promega UK Ltd. Southampton, UK), and 0.1 M of each primer. The thermocycling conditions were as follows: initial denaturing of 95 °C/5 min, followed by 35 cycles of 95 °C/30 s, 50 °C/30 s and 72 °C/1 min with a final extension at 72 °C/10 min. The product of the PCRs was visualized on TBE agarose gel (1% w/v) to assess the quality of amplification before pooling the triplicate reactions.

PCR purification and 16S rRNA gene sequencing

The PCR products were recovered from the agarose gel, pooled into a single sample per treatment, and purified using a QIAquick gel extraction kit (Qiagen). The samples were quantified using a Quant-iT Broad-Range DNA Assay Kit (Invitrogen) and BioTek Synergy HT microplate reader and combined in equimolar ratios. The 16S rRNA gene fragments were sequenced using Ion Torrent™ semiconductor technology chemistry. Template preparation was performed using an Ion OneTouch 2 System and Ion PGM Template OT2 400 Kit, and sequenced using Ion PGM Sequencing 400 on an Ion PGM System using an Ion 316 Chipv2.

Sequence data processing

The 16S rRNA reads were analyzed using mothur version 1.33.2 (Schloss, 2010) with a custom Snake make workflow (Koster & Rahmann, 2012). In summary, multiplexed reads were trimmed allowing 1 barcode mismatch and 2 primer mismatches, maximum homopolymer stretch of 8, minimum length of 250 bp, maximum length of 290 and Phred quality score >25. After trimming and filtering, chimeric sequences were removed. OTUs were aligned and classified with a confidence threshold of 80% using the Silva reference database (Quast *et al*., 2013) (release SSU_Ref_119). Sequences classified as chloroplasts and mitochondria were removed.

Statistical analysis

The BIOMfile with abundance and taxonomic information was created in mothur and the package 'phyloseq' (McMurdie& Holmes 2015) was used for importing the dataset into the R environment (Team *et al*., 2005). The analysis was carried out at phylum and genus taxonomical levels. To estimate if limited sampling affected the entire sampled population the Good's coverage estimator (Good 1953) was calculated. Alpha diversity was calculated using Renyi diversity profiles (Kindt and Coe, 2005), which ranks the samples according to relative diversity. Alpha diversity analysis was performed at the same level of surveying effort (2,197 sequences per sample) to avoid biases from different library sizes. One-way analysis of variance (ANOVA) was performed on diversity indexes (Species richness, Shannon, Simpson and Berger-Parker diversity index) depicted in the Renyi profiles. Further, analysis of phylum and genus abundances between treatments was performed using one-way ANOVA. The data was log transformed only when the residuals failed to satisfy the normality. To assess significance, *post hoc* analysis using the

Scott-Knott test was conducted. One-way ANOVA was carried out using the 'stats' R package (Team *et al*., 2005) and the Scott-Knott test was carried out using the 'laercio' Rpackage (Silva 2010). Data clustering at genus level (relative abundance) was performed using 'hclust'in 'stats'packages using UPGMA algorithm based on Bray-Curtis distance implemented in the 'vegan' R package (Oksanen *et al*., 2007).

RESULTS

In vitro screening of *Pseudomonas* strains for PGP traits and their effects on lettuce seedling growth under greenhouse conditions

From the 54 strains evaluated, 32 (59%) produced at least one of the evaluated compounds and 10 (18%) produced all of them (Table S1). In greenhouse experiments based on dry mass plant yields, 27 (50%) and 26 (48%) of the strains improved lettuce seedling shoot and root biomass, respectively, and 14 (26%) of the strains improved growth of the whole plants. Based on these results, we selected 12 of the 54 strains for further study (Table 1). Unlike the 12 selected strains, the IAC-RBcr1 and IAC-RBcr3 strains did not produce any of the metabolites evaluated and also did not improve seedling growth. Both strains were chosen as negative controls to confirm their ineffectiveness in for plants grown under field conditions. Strain IAC-RBal2 did not produce any of the metabolites evaluated, but still improved growth of the whole plants. Although the 12 selected rhizobacteria strains were isolated from different host plant species (chrysanthemum, rucola, and maize), they were equally effetive at improving the growth of lettuce plants.

16S rRNA gene and *nifH* analyses of selected rhizobacteria strains

Phylogenetic analysis of the 16S rRNA gene confirmed that the 12 strains were closely related to various known *Pseudomonas* species (Fig. 1). The IAC-RBmi1 strain was most similar to *Pseudomanas guariconensis*, while IAC-RBal1 and IAC-RBal2 strains were more similar to different *Pseudomonas* species. The strains IAC-RBcr6, IAC-RBcr3, IAC-RBcr2 and IAC-RBcr1 grouped with *Pseudomonas pleocoglossicida*. IAC-RBal3 strain grouped with *Pseudomonas parafulva*, while IAC-RBal4, IAC-RBcr4 and IAC-RBcr5 strains grouped with *Pseudomonas*

oryzihabitans and *P. cremoricolorata*. The IAC-RBru1 strain grouped with *Pseudomonas fluorescences*. All sequences are deposited in GenBank under the accession numbers listed in Table 1.

The *nifH* sequence could not be amplified using the Pol primers in any of the 12 strains, but positive detection of *nifH* was verified for the positive controls *R. leguminosarum* and *Pseudomonas* sp.

Rhizobacteria lettuce seedling and adult plant growth promotion under field conditions

In experiment 1, the seedlings inoculated with strains IAC-RBal2, IAC-RBal3 and IAC-RBcr2 yielded lower shoot dry mass than seedlings inoculated with the remaining 9 strains (Table 2). During plant development, the shoot dry mass was not significantly different with respect to the different treatments. However, the strains IAC-RBal2, IAC-RBal3, and IAC-RBcr5 improved root growth of the adult plants. The rhizospheric soil of plants inoculated with the strains IAC-RBcr2 and IAC-RBcr5 showed higher values of microbial biomass carbon (MBC) and basal soil respiration (BR) than other treatments, including the controls.

In experiment 2, seedlings treated with stains IAC-RBmi1 and IAC-RBcr1 yielded the highest values for shoot dry mass, but both of these strains had no effect on plant growth under field conditions. The treatment Mix (composed of the three strains IAC-RBmi1, IAC-RBcr1 and IAC-RBcr3) improved shoot dry mass of adult plants; however, the best effect was observed for plants inoculated with strain IAC-RBcr3, which improved growth of the whole plant. The various treatments in this experiment had no effect on MBC and BR of the rhizospheric soil.

In experiment 3, plants inoculated with strains IAC-RBcr4 and IAC-RBcr6 exhibited the highest shoot growth promotion (Fig. 2). Moreover, the in-field results obtained revealed that plants inoculated with IAC-RBru1 and IAC-RBcr4 strains reached the highest values of dry mass.

Surprisingly, all individual strains promoted root development, but not when they were applied incombination (Mix: IAC-RBcr4, IAC-RBcr6, IAC-RBru1 and IAC-RBal4). None of the individual strains nor the mix caused effects on rhizospheric MBC and BR. The strains used for experiment 3 exhibited the best effects on plant growth promotion compared to the results for experiments 1 and 2. Therefore, we sequenced the 16S rRNA genes of the rhizospheric soils for samples from experiment 3 to assess the impact of the inoculated strains on the rhizosphere bacterial community.

Impact of *Pseudomonas* strains on rhizosphere bacterial community

Rhizosphere microbiomes were assessed by 16S rRNA gene high throughput sequencing. After quality filtering, a total of 540,887 sequences were obtained with an average of 30,049 sequences per sample (minimum length 250 bp, maximum length 290, quality score >25). The average of sequencing coverage (Good's coverage) was 0.99 at genus taxonomic level (Supporting Table S4), indicating that the number of sequences from the libraries were adequately representative of rhizosphere microbial communities. The sequences are available at the European Nucleotide Archive (ENA) under the accession number PRJEB12505. Richness and evenness were determined by the Rényi diversity profiles (Fig. S1A). The control samples presented the same diversity measurements as the experimental treatments. The shape of each profile indicates the evenness. For example, a completely horizontal profile indicates that all species have the same evenness. According to the Shannon and Simpson indices, there were no significant differences in diversity between the various treatments. Furthermore, the dendrogram showed that the control and treatments did not cluster separately, suggesting no shift in bacterial community (Fig. S1B).

Sequences of 16S rRNA genes consistent with a total of 36 phyla, 78 classes, 149 orders, 274 families, and 452 genera were detected in the rhizosphere bacterial communities. An investigation of the twenty most abundant phyla revealed that in all treatments inoculated with *Pseudomonas* strains, members of *Firmicutes* and *Acidobacteria* were prominent, corresponding to a relative abundance of over 60% (Fig. 3). Plants inoculated with *Pseudomonas* strains clearly exhibited higher relative abundances of species belonging to *Acidobacteria* phyla. Remarkably, the abundances of *Proteobacteria* and *Chloroflexi* were lower for inoculated plants compared to non-inoculated plants.

To gain insight into the effects *Pseudomonas* species on the composition of lettuce rhizosphere bacterial communities, we performed a Scott-Knott test comparing all treatments. The results show that rhizosphere of plants treated with the IAC-RBru1 strain show a significant increase in the abundance of phyla *Planctomycetes*, *Verrucomicrobia*, *Candidate div BRC1*, *Armatimonadetes* and *Nitrospirae* (Table 3). Significant increases in abundances of the same phyla, except *Planctomycetes* were also observed for rhizospheres of plants inoculated with the IAC-RBcr4 strain. Interestingly, both IAC-RBru1 and IAC-RBcr4 strains showed the best results on growth promotion of field-grown lettuce.

Investigation of sequencing results at the genus level revealed *Bacillus* as the most abundant genus with at least 20% relative abundance in all treatments , followed by *Sphingomonas*, *Alicyclobacillus*, *Ralstonia* and *Bradyrhizobium* genera (Fig. 3). Inoculation with IAC-RBcr4 and IAC-RBru1 strains exhibited the most striking effects on the bacterial community composition, significantly raising the relative abundance of some genera (Table 3). For example, plants treated with the IAC-RBcr4 strain showed increased relative abundance of the genera *Pirellula*, *Fonticella*, *Anaerolinea* and *Isosphaera*, and plants treated with the RBru1 strain also showed increases in the relative abundances of *Anaerolinea* and *Isosphaera* as well as *Singulisphaera*, *Lentibacillus*, *Kribella* and *Siphonobacter*. Both IAC-RBcr4 and IAC-RBru1 strains had more impact on the rhizosphere bacterial community than the other strains we studied.

DISCUSSION

Pseudomonas characteristic traits and effects on lettuce seedling growth under greenhouse conditions

Some strains that produced IAA were additionally capable of producing HCN and solubilizing phosphate, all of which may have contributed to lettuce growth promotion. These characteristics are well described as plant growth promoting traits for various host crop plants including lettuce (Salamone *et al*. 2010; Sharma *et al*. 2011; Da Costa *et al*. 2013, Cipriano *et al*. 2013). However, the fact that some strains had the ability to improve the lettuce growth without producing any of the growth promoting metabolites investigated in the present study is not surprising. It was previously shown that growth promoter candidates do not necessarily display strong *in vitro* relationships between metabolite production and growth promoting effects (Ibañez *et al*. 2014). The beneficial effect may actually be triggered by another mechanism not evaluated here or that has not yet been identified. There are many other mechanisms through which PGPR can trigger plant growth promotion including, for example, classical siderophore production or synthesis of hydrogen sulfide, newly discovered as a plant growth promoter (Weger *et al*. 1988; Dooley *et al*. 2013; Gupta *et al*. 2014). Among the 12 selected *Pseudomonas* strains, only four were originally isolated from lettuce rhizospheres. Alternatively, six strains were obtained from chrysanthemum, one from rucola and one from maize rhizospheres, implying that non-host specific strains can mutually benefit different plant species. Also it is important to note that nitrogen fixation is a beneficial feature of the *Pseudomonas* group described in the literature (Rodríguez-Blanco *et al*. 2015). However, we were not able to amplify the *nifH* using DNA samples from the 54 *Pseudomonas* strains of the present study.

Pseudomonas strain effects on seedlings and adult plants

The phylogenetic analyses of 16S rRNA gene sequences revealed that the detected strains are closely related to species recognized as potential plant growth promoters. The IAC-RBru1 strain possessed a 16S rRNA sequence highly similar with that of *P. fluorescens*, a well-known lettuce growth promoter (Sundh *et al*. 2011) and described as a pathogen suppressor able to produce biofilm and phytoextraction activity (Andreote *et al*. 2009; Rokni-Zadeh *et al*. 2012; Zhang *et al*. 2012).

In experiment 1, inoculation with IAC-RBal2, IAC-RBal3 and IAC-RBcr5 strains did not promote growth in greenhouse-grown lettuce during the seedling phase, but significantly affected plant roots under field conditions. The IAC-RBal2 and IAC-RBal3 strains did not impact the microbial biomass (MBC) of the rhizospheres. However,change in the basal respiration (BR) in the rhizosphere due to inoculation of IAC-RBal2 strain suggests that plant root growth promotion occurred due to the inoculation without increasing the microbial activity. In contrast, the IAC-RBcr5 strain improved root growth in addition to increasing rhizospheric MBC and BR values. These results strongyl suggest that *Pseudomonas* strains belonging to the same species and obtained from the same plant species can trigger different responses in the host plant.

In experiment 2, the IAC-RBmi1 strain improved the growth of seedlings, but not in the later stage of plant development under field conditions. The plant growth promotion of adult plants due to the inoculation of IAC-RBcr3 strain manifest as improved growth of the entire plant, without significantly affecting biological MBC and BR rhizospheric properties. The results obtained for rhizospheric MBC and BR are in agreement with the study of Fließbach *et al*. (2009), who evaluated soil amendment with *Pseudomonas* and found no effect on MBC and BR after sixty days of plant cultivation (Fließbach *et al*. 2009). That MBC and BR values of rhizospheric soils treated with rhizobacteria influenced plant development at the beginning and at later stages of development has also been reported in independent studies (Zhen *et al*. 2014; Song *et al*. 2015). Furthermore, plants in soils with high fertility inoculated with beneficial strains remained relatively unaffected, while the effects of strains

in rhizospheric soil with lower fertility was found to be more pronounced (Fließbach *et al*. 2009).

The most surprising result of this study was that for experiment 3, in which all *Pseudomonas* strains individually enhanced plant root growth under field conditions, whereas the multistrain Mix treatment had little enhancing effect on growth. This result reveals novel information regarding plant growth promoting traits, namely, that strains with different characteristics are capable of promoting plant growth in different phases of plant development as indicated by the results for strains IAC-RBcr4 and IAC-RBcr6. Furthermore, this result is the opposite to those of numerous studies that have shown an inconsistent effect of growth promoting bacteria on plant cultivation (Weller 1988; Mark *et al*. 2006). The inconsistent effects of PGPR in those studies might be attributed to both abiotic and biotic factors. Temperature can change microbial community (Allison *et al*. 2008), especially under field conditions. In this study, the thermal amplitudes of daily temperature swings recorded in field experiments were high, ranging in average from 22.3 to 18.4 $^{\circ}$C (Table S3), and may have had an effect on colonization of *Pseudomonas* in rhizospheres. In particular, the high temperatures in experiments 1 and 2 may have had a large influence on the results. *Pseudomonas* are, in addition to being prominent in the rhizosphere, also well-known endophytes with documented effects on plant metabolism. These include several effects on plant hormones and defense compounds, which can reshape the plant associated (both endo- and rhizospheric) microbiome (Hallmant *et al*. 2006; Hardoim *et al*. 2015; Silveira et al. 2016). As we inoculated the seedlings with the various study strains, it is highly likely that these strains had effects on plant metabolism by altering the composition of root exudates, indirectly shaping the rhizosphere microbiomes. Similarly, the contrasting PGPR impacts of the same strains measured in a greenhouse setting (*i.e.* seedlings) versus determined under field conditions could be due to different plant colonization patterns of the experimental strains at different plant developmental stages. The consistent positive results of *Pseudomonas* inoculation for seedlings might be attributed to low competition of the strains with low numbers of resident microbial

community species present in the commercial growth substrate. We would expect this effect to be enhanced when large numbers of *Pseudomonas* cells are inoculated directly on seedlings.

We found that a mixture of strains promoted plant shoot growth under field conditions in experiment 2, but not in experiment 3. Several studies have investigated the effects due to a mixture of bacterial species, but many of them evaluated combinations of phylogenetically distant microorganisms such as *Pseudomonas* and *Azospirillum* or *Bacillus*, or growth promoters such as fungi. The idea of applying a mixture of bacteria seemed attractive to include in this study because microorganisms with different abilities might promote plant growth synergistically. For example, a mixture of *Bacillus* and *Staphylococcus* species has been shown to increase soybean seed yield. Similarly, the combination of *Pseudomonas* with *Bacillus* and *Rhizobium* promoted plant growth and decreased plant disease for legumes (Dutta *et al*. 2014; Prakamhang *et al*. 2015). However, there are reported cases of unsuccessful plant growth promotion due to the inhibition of a beneficial mechanism of a PGPR by other growth promoting species in the mixture (*e.g.* loss of biofilm production) (Kang *et al*. 2014). The mixture of different strains from the same *Pseudomonas* genus has not been commonly reported, especially for lettuce cultivation. However, Pierson and Weller (1994) evaluated the effects of various pseudomonad combinations and concluded that certain combinations increase the yield, whereas other combinations have negative effects on wheat plant yields (Pierson and Weller 1994). According to the authors, the high diversity of the strains introduced in the rhizosphere, in turn, resulted in a more diverse rhizosphere community that was able to colonize roots and survive biological, chemical, and physical changes occurring in the rhizosphere. In the present study, for the first time, we evaluated the effect of *Pseudomonas* co-inoculation in lettuce cultivated under field conditions and obtained examples of both success and failure in experiments 2 and 3, respectively. Further detailed studies will be necessary to assess the specific co-inoculation effects of these rhizobacteria on lettuce.

Pseudomonas strains impact on the rhizosphere microbial community

In general, *Firmicutes* was the most prominent phylum detected in our study, followed by *Acidobacteria*, *Actinobacteria* and *Proteobacteria*, in agreement with previous studies of lettuce rhizospheres under rhizobacteria influence (Schreiter *et al*. 2014a; Kröber *et al*. 2014). However, previous studies with rhizobacteria inoculants did not reveal a remarkable increase in the relative abundance of *Acidobacteria* as we observed in the present study. *Acidobacteria* was recently reported to be a new plant growth promoting bacterial phylum (Kielak et al. 2016). Lettuce inoculated with various strains of *Pseudomonas*, under field conditions, showed an increase in *Acidobacteria* and decrease of *Proteobacteria* phyla when compared to controls. All the inoculated strains, except IAC-RBcr6, were phosphate solubilizers (Table 1). Although we did not evaluate this capacity in the rhizosphere, these strains may possibly also possess this activity. Phosphate solubilization by microorganisms can be achieved by decreasing the pH (Marschner *et al*. 2011), which can favor growth of specific microbial groups such as *Acidobacteria* while not favoring growth of other groups, such as those mentioned above. The IAC-RBcr4 and IAC-RBru1 strains had significant effects on less abundant phyla such as *Verrucomicrobia*, *Candidate division* RBC1, *Plantomycetes*, *Armatimonadetes*, and *Nitrospirae*, which have not been described until now as component species of the rhizosphere. These findings led us to reject our initial hypothesis that*Pseudomonas* strains do not impact the microbial community in the rhizosphere of lettuce grown under field conditions.

Lettuce plants were cultivated in soil fields subjected of solarization before plant cultivation. Solarization has been described to have a positive impact on lettuce production in Brazil by negatively affecting plant pathogens and weeds (Patricio *et al*. 2006). Moreover, this method could impact the bacterial community by decreasing the microbial diversity and favoring groups resistant to high temperatures, such as

Firmicutes, which are capable of producing endospores (Weller and Wu 2015). Indeed, our results support this hypothesis since members of the *Firmicutes* phylum were among the most abundant for all treatments in this study. In addition, the consistently positive effects of inoculants on lettuce growth under field conditions might be because of the low competition of *Pseudomonas* inocula with low numbers of resident microbes remaining in solarized soil. Future studies on the possible advantages of solarization practice used in conjunction with PGPR promotion should be undertaken.

Another important finding here was that the pseudomonad strains inoculated on lettuce seedlings were found, after field growth, not to be among the most abundant genera comprising the rhizosphere. We expected to find representatives of *Pseudomonas* because of the high number of cells inoculated on the study plants and as this group is quite often reported to be enriched in lettuce rhizospheres due to exudation of organic acids including malate, fumarate, succinate and benzoate (Neumann *et al*. 2014). The pseudomonad group is described as being capable of using these acids as sole carbon sources and is known to exhibit chemotactic behaviors towards concentration gradients of these compounds (Fuente *et al*. 2007; Oku *et al*. 2014). However, our results are from samples collected four weeks after inoculation, a period of time that likely allowed for interactions between the endogenous microbes and the applied test strains that could alter the respective fractional populations. Our results corroborate the previously published finding of *Bacillus* as the most abundant genus detected in lettuce rhizosphere inoculated with *Pseudomonas* (Schreiter *et al*. 2014b), although the soil in that study had not been solarized. *Bacillus* can be recruited by malic acid exudation from plants (Rudrappa *et al*. 2008), as is known to be the case for lettuce (Neumann *et al*. 2014).

The second most abundant genus detected in our study was *Sphingomonas* (phylum Proteobacteria), which also has been described as one of the most abundant genera in lettuce rhizospheres (Schreiter *et al*. 2014c). When inocula were applied to the rhizospheres, especially IAC-RBcr4

and IAC-RBcr1 inocula, we also observed significant increases in the relative abundance of *Acidothermus* (phylum Actinobacteria) and other less abundant genera (Table 3).

In the present study, we significantly gained practical knowledge about the interaction between *Pseudomonas* and lettuce by including field experiments. Our study results demonstrated a positive effect on lettuce seedling and adult plant growth due to the inoculation of *Pseudomonas* strains under field conditions. In summary, IAC-RBcr4 and IAC-RBru1 strains improved lettuce production up to 30% (total dry mass) without counterproductive effects on the rhizospheric microbial community diversity. Sequencing analysis of the rhizospheric bacterial community revealed that the strains we investigated favored enrichment in the abundances of *Isosphaera*, *Pirellula* (phylum Planctomycetes), *Acidothermus*, *Pseudolabrys*, *Singulisphaera*, *Bribella*, *Fonticella* (phylum *Actinobacteria*), *Lentibacillus* (phylum *Firmicutes*), *Anaerolinea*, *Elstera* (phylum *Chloroflexi*), and *Siphonobacter* (phylum *Bacteroidetes*). The role of these microorganisms in lettuce rhizospheres has not yet been described and should be investigated in future studies.

Previously, in many studies of PGPR, reports of the beneficial effects on the host plant have been inconsistent. Our study is the first to show a consistent, reproducible effect of *Pseudomonas* strains on lettuce growth promotion in seedling and mature plant grown under field conditions. Moreover, this study combined the results of field experiments (conducted with the same management used by local lettuce producers) with next generation sequencing analysis to better characterize *Pseudomonas* inoculant use in sustainable lettuce production.

Conflict of interest statement

The authors declare no conflicts of interest.

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Table 1. Characteristics of *Pseudomonas* strains chosen for molecular characterization and field experiments, including plant growth promoting (PGP) traits and dry mass increase (+) or not (-) and 16S RNA gene sequence accession numbers deposited in GenBank.

		PGP traits			Dry mass		
Strains	Origin host plant	IAA[a]	HCN[b]	PS[c]	Shoot	Root	Accession number
IAC-RBcr1	Chrysanthemum	-	-	-	-	-	KJ590496
IAC-RBcr2		-	-	-	+	+	KJ590497
IAC-RBcr3		-	-	-	-	-	KJ590498
IAC-RBcr4		+	+	+	+	+	KJ590499
IAC-RBcr5		+	-	+	+	-	KJ590500
IAC-RBcr6		-	-	-	+	+	KJ590501
IAC-RBal1	Lettuce	+	+	+	+	+	KJ590502
IAC-RBal2		-	-	-	+	+	KJ590503
IAC-RBal3		-	+	-	+	+	KJ590504
IAC-RBal4		+	+	+	-	-	KJ590505
IAC-RBru1	Rucola	-	-	+	+	-	KJ590506
IAC-RBmi1	Maize	+	+	+	+	+	KJ590507

[a]Indole-3-acetic acid production; [b]hydrogen cyanide production; [c]phosphate solubilization

Table 2. Rhizobacteria strains and their effect on lettuce plants at different growth stages (seedlings and adult plants in field) and on the rhizospheric soil microbial biomass (MBC) and basal respiration (BR).

	Treatments	Seedlings		Adult plants		Rhizospheric soil	
		Shoots	Roots	Shoots	Roots	MBC	BR
Experiment 1	Control	0.77 a*	0.28 a	12.56 a	0.96 b	60 b*	68 c
	IAC-RBal1	0.42 c	0.26 a	12.27 a	0.98 b	118 b	53 d
	IAC-RBal2	0.47 c	0.19 a	12.63 a	1.10 a	106 b	48 d
	IAC-RBal3	0.60 b	0.23 a	12.95 a	1.05 a	88 b	73 c
	IAC-RBcr2	0.68 b	0.24 a	12.80 a	0.97 b	188 a	114 a
	IAC-RBcr5	0.40 a	0.19 a	12.69 a	1.08 a	216 a	104 b
Experiment 2	Control	0.42 c	0.16 a	8.91 b	0.46 b	160 a*	103 a
	IAC-RBmi1	0.62 a	0.18 a	9.62 b	0.49 b	120 a	109 a
	IAC-RBcr1	0.53 b	0.17 a	9.76 b	0.50 b	113 a	102 a
	IAC-RBcr3	0.45 c	0.16 a	10.19 a	0.56 a	112 a	117 a
	Mix	0.47 c	0.17 b	9.15a	0.47 b	83 a	115 a
Experiment 3	Control	0.40 b	0.14 a	8.56 b	0.41 b	310 a	68 a
	IAC-RBcr4	**0.59 a**	0.17 a	**11.20 a**	**0.53 a**	330 a	75 a
	IAC-RBcr6	**0.57 a**	0.18 a	9.53 b	**0.48 a**	317 a	67 a
	IAC-RBru1	0.47 b	0.16 a	**10.15 a**	**0.49 a**	401 a	80 a

	IAC-RBal4	0.43 b	0.15 a	9.09 b	**0.47 a**	319 a	81 a
	Mix	0.37 b	0.15 a	9.42 b	0.44 b	320 a	74 a

Evaluation of shoot and root dry mass (g/plant).*Treatments with the same letter do not differ by the Scott-Knott test ($P<0.05$). The data with the bold letters refer to the best result in different phase of lettuce development.

Table 3. Relative abundance of bacteria (phylum and genus levels) in rhizospheric soil of lettuce plants inoculated with different strains of *Pseudomonas* grown in field conditions.

Phyla	Control		IAC-RBcr4		IAC-RBcr6		IAC-RBru1		IAC-RBal4		Mix		Significance
Verrucomicrobia	1.6×10^{-3}◊	b[1]	3.4×10^{-3}	a	1.4×10^{-3}	b	3.4×10^{-3}	a	1.0×10^{-3}	b	1.4×10^{-3}	b	*[2]
Candidate div BRC1	2.0×10^{-4}	b	8.1×10^{-4}	a	2.6×10^{-4}	b	7.5×10^{-4}	a	9.4×10^{-5}	b	1.7×10^{-4}	b	*
Planctomycetes	12.4×10^{-4}	b	17.2×10^{-4}	b	11.5×10^{-4}	b	30.0×10^{-4}	a	7.3×10^{-4}	b	5.7×10^{-4}	b	*
Armatimonadetes	2.7×10^{-4}	b	4.4×10^{-4}	a	2.7×10^{-4}	b	4.8×10^{-4}	a	1.9×10^{-4}	b	2.5×10^{-4}	b	*
Pitropirae	2.0×10^{-4}	b	3.3×10^{-4}	a	1.9×10^{-4}	b	3.4×10^{-4}	a	1.6×10^{-4}	b	1.7×10^{-4}	b	****
Genus	**Control**		**IAC-RBcr4**		**IAC-RBcr6**		**IAC-RBru1**		**IAC-RBal4**		**Mix**		**Significance**
Acidothermus	6.1×10^{-4}	b	9.5×10^{-4}	b	11.0×10^{-4}	a	7.8×10^{-4}	b	6.0×10^{-4}	a	1.1×10^{-4}	a	**
Pseudolabrys	1.9×10^{-4}	b	1.2×10^{-4}	b	4.5×10^{-4}	a	1.6×10^{-4}	b	3.4×10^{-4}	a	2.7×10^{-4}	a	**
Singulisphaera	3.7×10^{-5}	b	3.8×10^{-5}	b	5.5×10^{-5}	b	10.2×10^{-5}	a	1.1×10^{-5}	b	3.7×10^{-5}	b	*
Reyranella	2.0×10^{-4}	a	1.0×10^{-4}	b	2.1×10^{-4}	a	1.3×10^{-4}	b	3.1×10^{-4}	a	2.4×10^{-4}	a	**
Lentibacillus	1.5×10^{-4}	b	1.8×10^{-4}	b	1.4×10^{-4}	b	4.5×10^{-4}	a	1.8×10^{-4}	b	3.2×10^{-4}	a	**
Anaerolinea	2.6×10^{-4}	b	7.77×10^{-4}	a	8.5×10^{-5}	b	1.4×10^{-3}	a	4.0×10^{-5}	b	8.6×10^{-5}	b	**
Elstera	1.1×10^{-4}	c	5.4×10^{-6}	d	1.7×10^{-4}	b	0	d	2.8×10^{-4}	a	9.7×10^{-5}	c	***
Isosphaera	8.9×10^{-5}	b	19.0×10^{-5}	a	7.6×10^{-5}	b	20.0×10^{-5}	a	3.3×10^{-5}	b	8.9×10^{-5}	b	*
Pirellula	7.0×10^{-5}	b	9.9×10^{-5}	a	6.8×10^{-5}	b	15.1×10^{-5}	a	2.6×10^{-5}	b	2.2×10^{-5}	b	*
Kribbella	5.9×10^{-5}	b	8.7×10^{-5}	b	1.2×10^{-4}	b	4.4×10^{-4}	a	1.3×10^{-4}	b	1.9×10^{-4}	b	**
Fonticella	11.5×10^{-5}	b	19.0×10^{-5}	a	7.5×10^{-5}	b	7.7×10^{-5}	b	6.7×10^{-5}	b	8.1×10^{-5}	b	****
CandidatusMicrothrix	6.2×10^{-5}	a	3.7×10^{-5}	b	8.4×10^{-5}	b	0	b	4.3×10^{-5}	a	3.7×10^{-5}	b	*
Defluviicoccus	5.2×10^{-5}	a	$8.0\ 10^{-6}$	b	8.1×10^{-5}	a	0	b	2.0×10^{-5}	b	5.1×10^{-5}	a	*
Thermoanaero-bacterium	2.0×10^{-5}	a	0	b	0	b	0	b	0	b	3.5×10^{-5}	b	****
Siphonobacter	0	b	0	b	0	b	6.6×1^{-5}	a	0	b	3.5×10^{-5}	b	****

◊Values are the means of triplicates.

[1]Values with the same letter are not significantly different based on Scott-Knott test.

[2]Significance levels: *P<0.01, **P<0.001, ***P=0, ****P<0.05

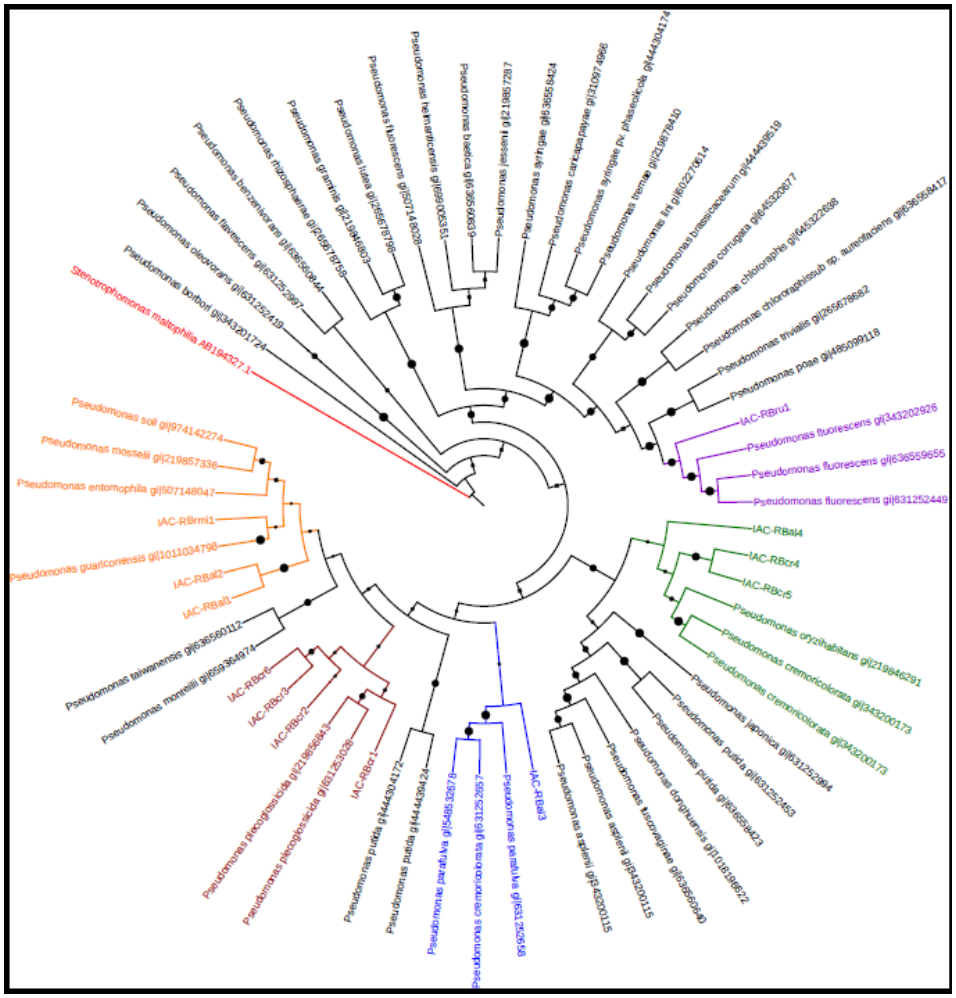

Figure 1. Phylogenetic tree based on the alignment of 16S rRNA gene sequences from selected rhizobacteria strains from this study and reference sequences from GenBank. Circles at the nodes indicate bootstrap support >75%. *Stenotrophomonas maltophilia* was used as the outgroup.

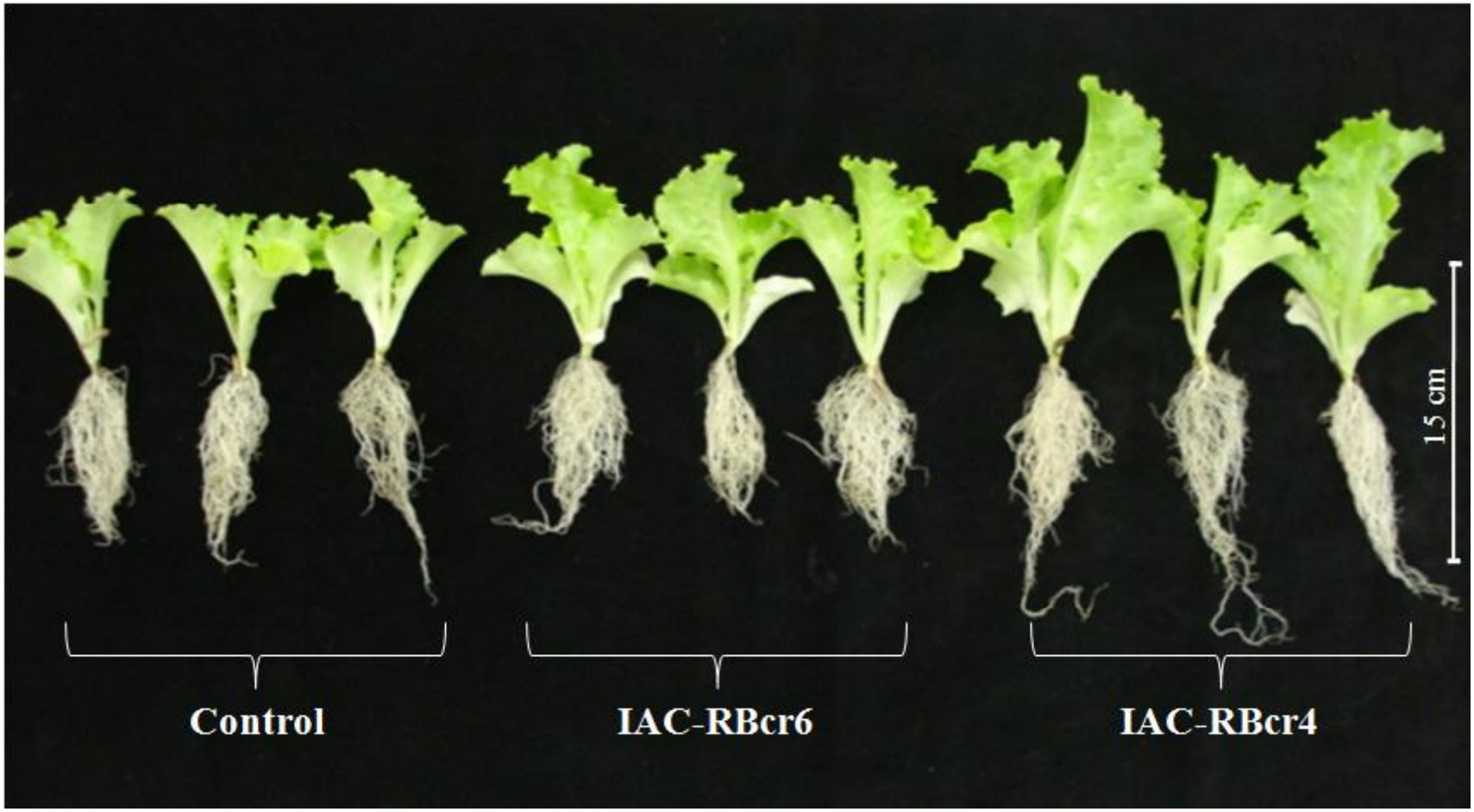

Figure 2. Four-week-old lettuce seedlings untreated (Control) or treated with two rhizobacterial strains.

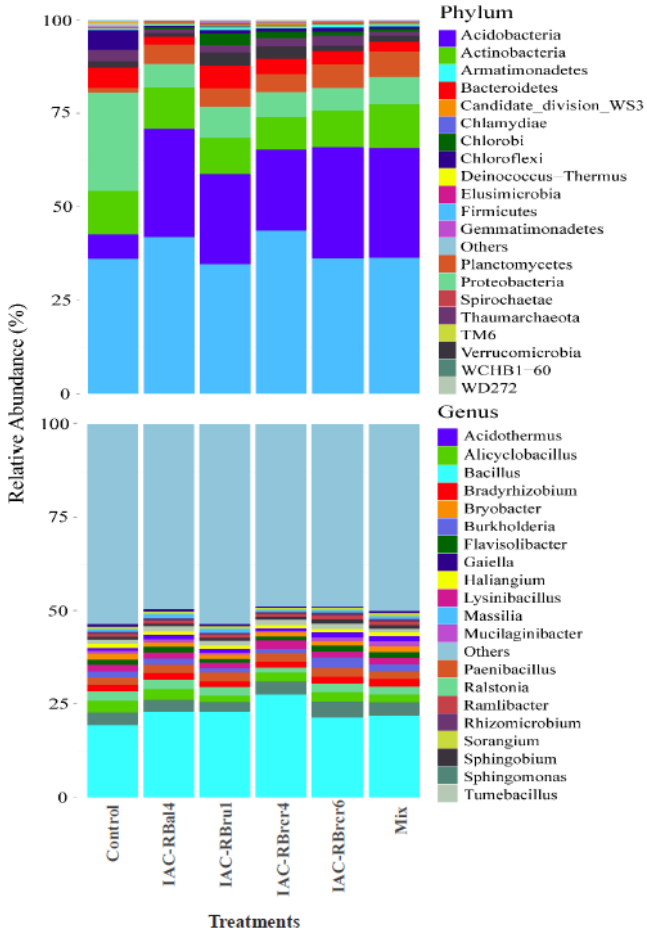

Figure 3. Stacked bar charts of the twenty most abundant phyla and genera in the rhizospheric microbial community of lettuce inoculated with various *Pseudomonas* strains. Each bar corresponds to the mean of triplicate determinations.