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Selection of the Root Endophyte *Pseudomonas brassicacearum* CDVBN10 as Plant Growth Promoter for *Brassica napus* L. Crops

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Abstract: Rapeseed (*Brassica napus* L.) is an important crop worldwide, due to its multiple uses, such as a human food, animal feed and a bioenergetic crop. Traditionally, its cultivation is based on the use of chemical fertilizers, known to lead to several negative effects on human health and the environment. Plant growth-promoting bacteria may be used to reduce the need for chemical fertilizers, but efficient bacteria in controlled conditions frequently fail when applied to the fields. Bacterial endophytes, protected from the rhizospheric competitors and extreme environmental conditions, could overcome those problems and successfully promote the crops under field conditions. Here, we present a screening process among rapeseed bacterial endophytes to search for an efficient bacterial strain, which could be developed as an inoculant to biofertilize rapeseed crops. Based on in vitro, in planta, and in silico tests, we selected the strain *Pseudomonas brassicacearum* CDVBN10 as a promising candidate; this strain produces siderophores, solubilizes P, synthesizes cellulose and promotes plant height in 5 and 15 days-post-inoculation seedlings. The inoculation of strain CDVBN10 in a field trial with no addition of fertilizers showed significant improvements in pod numbers, pod dry weight and shoot dry weight. In addition, metagenome analysis of root endophytic bacterial communities of plants from this field trial indicated no alteration of the plant root bacterial microbiome; considering that the root microbiome plays an important role in plant fitness and development, we suggest this maintenance of the plant and its bacterial microbiome homeostasis as a positive result. Thus, *Pseudomonas brassicacearum* CDVBN10 seems to be a good biofertilizer to improve canola crops with no addition of chemical fertilizers; this the first study in which a plant growth-promoting (PGP) inoculant specifically designed for rapeseed crops significantly improves this crop's yields in field conditions.

Keywords: *Pseudomonas*; PGPB; bioinoculants; endophytes; bacterial microbiome; culturome; genome sequencing; *Brassica napus*

1. Introduction

The FAO estimates that there will be 2.3 billion more people on the Earth in 2050, in a world already struggling to combat poverty and hunger. Thus, we need to increase our capability to produce food using the limited natural resources of our planet more efficiently while fighting climate change. Chemical fertilizers increase crop yields, but they have negative effects for human and animal health, contaminate soils and water, and their fabrication, which requires huge amounts of energy, contributes to resource depletion and global warming [1]. Moreover, the excessive or repetitive use of chemical fertilizers usually presents low efficiency in their use by plants because the soil biogeochemical cycles is often altered [2].

Alternatively, plant growth-promoting bacteria (PGPB), which are naturally occurring microbes that modulate plant growth due to their metabolic activities, can enhance crop yields when applied as biofertilizers [3–5]. PGPB can fix atmospheric nitrogen, produce siderophores and/or phytohormones, solubilize phosphorous and/or potassium and inhibit the growth of pathogenic microorganisms [6]. Within PGPB, endophytes are particularly interesting because, once inside the plant, they do not need to compete with the dense population of bacteria in the rhizosphere and they are protected from extreme abiotic conditions, so they have more chances to succeed when applied in the fields [7,8].

Endophytes are part of the plant microbiome and play essential roles for its fitness and survival [9]. Many of these microorganisms are non-cultivable in routine laboratory conditions and thus, culture independent methods allow us to unravel the complete microbial diversity living within the plants. These endophytic microbiomes, as occurs in animals, interact with their host in essential functions [10–12]; hence, plant microbiome research highlights the importance of indigenous microbial communities for host phenotypes such as growth and health [13].

Brassica napus L. (rapeseed, canola) is an important crop due to its cultivation not only as a food resource (human and animal), but also for biodiesel production, being one of the most significant oilseed crops in temperate climates [14]. In Europe, rapeseed seeds are the primary source of oil for biodiesel production, its by-product being a high protein source for animal feeding [15]. However, rapeseed cultivation requires important amounts of chemical fertilizers [16], and therefore, alternatives that enable the reduction in chemical fertilization for a more sustainable crop are very desirable. This implies the use of biofertilizers, which include endophytic PGPB.

Thus, the design of an efficient bacterial endophytic inoculant for rapeseed crops which could increase rapeseed crop yields with no addition of chemical fertilizers is very desirable. For that purpose, it is necessary to study the members of the bacterial endophytic population, those members of the endophytic community which can be artificially cultured and thus biotechnologically produced and formulated.

In terms of plant growth-promoting (PGP) functionality, in vitro PGP mechanisms have been analyzed in just a few rhizospheric [17] or endophytic bacteria associated with *B. napus* plants [18,19]. In addition, the information about the effects of PGPB in rapeseed plants is scarce [18,20–23]. Taking advantage of next generation sequencing, PGPB genome sequence annotation and analysis allow in silico studies of the genetic potential of a bacterium to promote plant growth, including the discovery of specific PGP traits and/or pathways, such as tolerance to different biotic and abiotic stresses, heavy metal detoxifying activity or biological control potential [24].

These massive parallel sequencing techniques are becoming even more interesting when applied to elucidate the taxonomic composition and biological functions of the plant and soil microbiome when plants grow under field conditions, where they can be used to recreate the microbial communities' dynamics [25].

Based on the hypothesis that bacterial endophytes can be efficient biofertilizers when applied as inoculants in the fields, the aim of this work was to isolate and select a rapeseed bacterial endophyte with the potential to promote rapeseed growth and yields. For that, we obtained a collection of rapeseed endophytic bacteria and analyzed the potential of our isolates as plant growth promoters, through a screening of a few in vitro PGP mechanisms followed by the analysis of the in planta effect

with several selected isolates, evaluating their capability to promote rapeseed seedling growth. Once we had selected the best-performing strains in planta, we obtained the genome sequences of the best PGP endophytic strains to deepen the study of their molecular machinery implicated in plant colonization and growth promotion. The in silico and in vivo assays allowed us to select one particular strain, which was inoculated in a field trial, showing for the first time a significant increase in rapeseed yields using a PGP bacterium inoculum. As a novelty, we analyzed the impact of the inoculation of the strain not only in the plant development and crop yields, but also on the root endophytic community.

2. Materials and Methods

2.1. Isolation and Identification of Bacterial Isolates

Rapeseed plants (*B. napus* cv rescator) in the phenological stage of rosette were collected in February 2017 from two agricultural soils located in the municipalities of Castellanos de Villiquera (CDV) (province of Salamanca) and Peleas de Arriba (PDA) (province of Zamora), both in Spain. Plants were extracted from the soils, kept refrigerated and shipped to the laboratory, where they were processed within two hours from the time of extraction.

To isolate rapeseed root bacterial endophytes, roots were excised carefully and washed in sterile Petri dishes containing sterile distilled water (× 10 times) and then surface-disinfected by immersion in sodium hypochlorite (2%) for 2 min. After that, surface-disinfected roots were washed 5 times in sterile distilled water and dried with sterile filter paper. An aliquot of water from the last washing step of each sample after the disinfection protocol and a few entire disinfected roots were plated as disinfection controls. No bacterial growth was observed in those plates.

Surface-disinfected roots were smashed in a sterile mortar and the content was serially diluted with sterile distilled water. Then, 100 µL of the 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated onto Petri dishes containing different media to target the isolation of a wider biodiversity: Tryptic Soy Agar (TSA; BD Difco, Franklin Lakes, NJ, USA), YMA (Laboratorios Microkit, Madrid, Spain), 869 medium (Tryptone (10 g/L), yeast extract (5 g/L), NaCl (5 g/L), D-glucose (1 g/L), $CaCl_2$ (0.345 g/L), and agar (20 g/L)) and ten times diluted 869 medium.

Plates were incubated at 28 °C for 21 days. The emerging bacterial colonies were regularly isolated to get pure cultures. Their names were composed by CDV or PDA, depending on the sampling origin, followed by BN, from *Brassica napus* and a correlative number. Then, isolated strains were stored in a sterile 20% glycerol solution at −80 °C for long-term storage.

For bacterial strain identification, DNA was obtained using the REDExtract-N-Amp™ PCR Ready Mix (Sigma-Aldrich Co. LLC), following the instructions given by the manufacturer. Then, strains were grouped at species or subspecies level based on their 879F-RAPD fingerprints, obtained as detailed by Igual et al. [26] and grouped by means of the UPGMA algorithm (unweighted pair grouping with mathematic average) using the software package BioNumerics version 4.5 (Applied Maths NV, Sint-Martens-Latem, Belgium), with a threshold of 75% similarity. To identify a representative bacterial isolate of each 879F-RAPD group, 16S rRNA gene sequences were amplified as described in Rivas et al. [27] and processed as described in Poveda et al. [28]. Nearly complete (~1500 bp) sequences were compared with those from type strains deposited in GenBank using BLASTn program [29] and EzTaxon tool [30].

In the case of those bacterial strains selected for genome sequences, housekeeping gene sequences (*gyr*B and *rpo*B) were retrieved from the genome and compared to those available in the GenBank database using BLASTn for a more accurate taxonomic identification.

In the case of the strain inoculated in the field trials, a phylogenetic analysis of the 16S rRNA gene sequence of the strain and those of the closely related species was done as detailed in Jiménez-Gómez et al. [31].

2.2. In Vitro Analyses of Plant Growth-Promoting Mechanisms and Biosynthesis of Polysaccharides

Bacterial siderophore production and solubilization of non-assimilable phosphates were evaluated as detailed in Jiménez-Gómez et al. [31]. Briefly, siderophore production was evaluated by inoculating in M9-CAS-agar medium plates [32] modified according to the suggestions given by Alexander and Zuberer [33]. The solubilization of non-soluble phosphates into soluble assimilable ions was analyzed in Pikovskaya medium plates [34], which contain bicalcium phosphate ($CaHPO_4$) or tricalcium phosphate [$Ca_3(PO_4)_2$] as the P source. Polysaccharide (cellulose and cellulose-like polymers) biosynthesis ability of each isolate was determined as described by Robledo et al. [35]. All plates were incubated for up to 21 days at 28 °C, recording the results every week. Nitrogen fixation was assayed in liquid medium as detailed in Poveda et al. [28]. The method shows the ability of strains to grow in a N-free minimal liquid medium. Indole acetic acid (IAA)-like compound production was measured by the colorimetric method described in Khalid et al. [36].

2.3. Effects of Bacterial Isolates on Rapeseed Seedlings

Rapeseed seeds (cv rescator) were surface-disinfected with 70% ethanol for two min, followed by soaking in an aqueous 5% sodium hypochlorite solution for ten minutes. Then, they were washed five times with sterile water and pre-germinated on water-agar plates (1.5%) for 24 h. To prepare the inoculum, bacteria were grown in their respective isolation media for 3 days at 28 °C; afterwards, the Petri dishes were flooded with saline buffer (0.9% NaCl) in order to obtain the cell suspensions, which were adjusted to an O.D. (600 nm) of 0.5, corresponding to final concentrations of ~10^8–10^9 CFU/mL (this concentration was determined after counting the number of viable cells using the serial decimal dilution method). After the pre-germination and inoculum preparation steps, Petri dishes containing the seedlings were inoculated. Twelve plates per treatment with five seeds per plate were prepared for the in vitro analyses. Thirty seedlings per treatment were collected at five and fifteen days post-inoculation, respectively. Values of seedling height and root length were recorded at each collection time.

2.4. Draft Genome Sequencing and Annotation

The genome sequence was obtained from selected strains after the plant growth promotion tests on rapeseed seedlings. For genome sequencing, the DNA was obtained from selected bacteria after two days of growth at 28 °C using the Quick DNA Fungal/Bacterial Miniprep kit (Zymo Research, Irvine, CA, USA) following the procedure described by the manufacturer.

The draft genome of selected isolates was sequenced on an Illumina MiSeq platform as described by Saati-Santamaría et al. [37]. The sequence data were assembled using Velvet (v1.12.10) [38]. Gene calling, annotation, and search for genes related to plant growth promotion- and colonization-related capabilities was performed using RAST (v2.0) pipeline [39] and then re-checked by BLASTp against known conserved proteins from phylogenetically related or closest relatives *Pseudomonas* strains. The Genome Shotgun project for strains CDVBN10 and CDVBN20 has been deposited at DDBJ/EMBL/GenBank under the accessions VDLV00000000 and VDLW00000000, respectively. The versions described in this paper are versions VDLV01000000 and VDLW01000000.

2.5. Field Experiment

The most promising PGP bacterium according to *in vitro*, *in vivo*, and *in silico* experiments, strain *Pseudomonas brassicacearum* CDVBN10, was assayed in field conditions as a rapeseed biofertilizer.

The field trial was performed between September 2018 and May 2019 in the locality of Cañizal (Zamora; NS/EW coordinates: 41.152627/-5.356508). The field has a crop history of rotations between sunflower and barley. No rapeseed crops were sown previously in this soil. The soil is a non-saline soil with loamy-sandy texture, with a good organic matter content (5.6%), showing a very slightly basic pH (7.87) and a low EC (EC1:2 0.096 dS/m). The electrical conductivity and the pH were measured according to Dellavalle [40]. The mineral content of the soil is as follows: total N < 0.045%; assimilable

P 15 mg/kg, K 0.25%, Zn 21.9 mg/Kg, Fe 1.2%. The number of colony formation units (CFU) per gram of soil (counted in Plate Counting Agar (PCA; Sigma-Aldrich Co. LLC, St. Louis, MO, USA) plates incubated at 28 °C for 7 days) is 1.2×10^7.

The experimental field was divided in six rows with 5 m length by 2 m width (10 m^2) with a 0.5 m buffer non-cropped area between them to avoid the transfer of bacteria between plots. Plants were grown in a density of 12 plants per linear meter in each row and they were rainfed.

The experiment was arranged in a randomized block design with three replicates per treatment. No chemical fertilization was applied to the soil. One month after the seeding, once the seedlings had emerged, a bacterial suspension with a cell density of 10^9 CFU/mL was prepared on sterile saline buffer (0.9% NaCl), using 3 day old bacterial cultures grown at 28 °C in TSA. A total of 5 mL of the bacterial suspension was added to each plant. For uninoculated control, equal volume of sterile saline buffer was added per plant. Fifteen days after the inoculation of the plants, the application was repeated.

The rapeseed plants were collected at seed maturity stage, approximately 8 months after seeding. Thirty randomly selected plants of each plot were harvested and kept separately. Plants were quickly taken to the lab on ice, where we separated roots from shoots carefully. Roots were excised for further amplicon sequencing. From each plant, grain yield and total shoot dry biomass (oven-dried at 60 °C) were recorded. Dry plants were also used for the analysis of N, C, Fe, P, K at the Ionomics Service at CEBAS-CSIC (Murcia, Spain), using an Elemental Analyst model TruSpec CN628 equipment (Leco, St Joseph, MI, USA) for the N analysis, and ICP THERMO ICAP 6500DUO equipment (Thermo Fisher, Waltham, MA, USA) for the analysis of the remaining elements.

2.6. Amplicon Sequencing and Sequence Analysis

Total genomic DNA was obtained from rapeseed roots collected as explained in the previous section using the DNeasy Power Plant Pro Kit (Qiagen®, Venlo, Netherlands), following the instructions given by the manufacturer. For each location, DNA from roots of three different plants of each treatment was pooled and amplicons of the complete bacterial 16S rRNA gene (V1-V9 regions) were sequenced on a PacBio Sequel system using a SMRT Cell 1M V3 LR. PacBio circular consensus sequences (CCS) were used to obtain sequences with a low error rate in the consensus sequence resulting from the alignment between all the subreads from the same molecule.

Sequences with lengths ≥800 nt to ≤1600 nt were filtered using SEED2 software package [41]. QIIME (v1.9) software [42] was used for amplicon data analysis. The sequences were aligned and taxonomically classified (97% threshold) using the Greengenes 16S rRNA sequence database, release 13.8.97 [43] with an open-reference picking method for the OTU (Operational Taxonomic Units) clustering, using the default settings of the UCLUST algorithm. Chimeric sequences were removed using UCHIME (v6.1.544) [44]. Lineages belonging to chloroplast and mitochondria were removed with QIIME scripts. PacBio reads were deposited in NCBI under the SRA accession PRJNA601164.

Comparison between control and bacteria-treated samples and plots summarizing taxa were made following QIIME scripts. The alpha diversity was measured with the Phylogenetic Diversity (PD), Chao1, Shannon's, Simpson´s and Good´s coverage indexes. Comparisons between treatments were made using the Kruskal–Wallis statistic test [45] applying the Benjamini–Hochberg false discovery rate (FDR) procedure for multiple comparisons [46]. OTU tables were rarefied using the lower sequence count among all samples as maximum rarefaction depth. The beta diversity of the samples was measured using weighted and unweighted UniFrac distances. Beta diversity comparison of treatments was made through nonparametric *p*-values with the Bonferroni correction [47], calculated after 999 Monte Carlo permutations. A value of $p > 0.05$ was used as a threshold for statistical significance of OTU correlation to a control or treated samples.

2.7. Statistical Analysis of Plant Parameters

Statistical comparisons of plant growth assays, including parameters recorded of the plants collected from the field assay, were carried out using the StatView 5.0 (SAS Institute, Inc., Cary,

NC, USA) [48] and performed using one-way analysis of variance (ANOVA). P values of 0.05 or less ($p \leq 0.05$) were considered statistically significant. Fisher's protected least significant differences (LSD) test was used as post hoc test.

3. Results

3.1. Bacterial Culturome Shows the High Diversity of B. Napus Associated Endophytic Bacteria

Using a combination of rich and minimal media to target the isolation of a wider biodiversity, we obtained 112 bacterial isolates from surface-disinfected rapeseed roots collected in the same Spanish locations previously mentioned. From them, 31 strains were isolated from plants collected in PDA and 81 from plants collected in CDV (Table 1).

We used 879F-RAPD fingerprints to group the strains at infraspecific level in order to select representative strains for their identification. The 31 strains from the location of PDA (Zamora) clustered into 20 different 879F-RAPD groups, while the 81 bacterial isolates from the locality of CDV (Salamanca) clustered into 56 different groups (Table 1).

Afterwards, we chose a representative strain (marked in Table 1 with an asterisk) from each 879F-RAPD group to obtain its 16S rRNA gene sequence. Then, we compared the obtained sequences with those of the type strains of described species. The closest related species to each isolate is shown in Table 1. The bacterial community analysis of the culturable bacterial endophytes of the rapeseed roots of plant collected in the two agricultural lands of this study revealed the presence of 39 different species within 27 different genera (Table 1).

The dominant genera were *Pseudomonas*, *Pseudoarthrobacter* and *Bacillus*, with 49, 12 and 10 strains belonging to 29, 4 and 6 different 879F-RAPD groups, respectively. In addition, strains belonging to these three genera were found in plants cultivated in both locations of this study, while all the other genera were location specific.

3.2. In Vitro Analyses of Plant Growth-Promoting Mechanisms

The in vitro tests of PGP potential include the analyses of phosphorous (P) solubilization, siderophores production and cellulose biosynthesis.

The results of the in vitro analyses of the PGP traits performed in this study are summarized in Table 1. A total of 77.4% and 67.9% of the isolates associated with plants from PDA and CDV, respectively, solubilize phosphate. Concerning siderophores, 38.7% of the strains isolated from PDA showed siderophore production, whereas 55.5% of the bacterial isolates from CDV produced these iron-chelating molecules. Finally, more than half of the strains from this study showed capability to synthesize cellulose or cellulose-like polymers.

Regarding PGP traits of the bacteria selected for the in planta experiments, all strains but one synthesized IAA-like molecules, all but one solubilized tricalcium phosphate and only *Bacillus simplex* CDVBN6 was able to grow with no addition of a nitrogen source in the medium.

3.3. Plant Growth Promotion in Rapeseed Seedlings under Controlled Conditions and Additional PGP Traits

Those strains showing the best results in the in vitro test of PGP traits (grey-highlighted name in Table 1) were used to evaluate their PGP capability in planta, using rapeseed seedlings. These strains were also assayed for IAA-like production, nitrogen fixation and $Ca_3(PO4)_2$ solubilization. The results for the PGP ability of these strains are summarized in Table 2.

Table 1. Identification of strains isolated in this study and in vitro plant growth-promoting mechanisms.

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
CDVBN92A	869 1/10	I	*Pseudarthrobacter oxydans* ATCC 14358^T	-	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN98 *	869 1/10	I	*Pseudarthrobacter oxydans* ATCC 14358^T	99.58	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN100 *	869 1/10	II	*Isoptericola nanjingensis* H17^T	97.42	Actinobacteria, Actinobacteria, Micrococcales, Promicromonosporaceae			
PDABN24A *	YMA	IV	*Dermacoccus nishinomiyaensis* DSM 20448^T	99.35	Actinobacteria, Actinobacteria, Micrococcales, Dermacoccaceae			
CDVBN92B *	869 1/10	V	*Agromyces ramosus* DSM 43045^T	99.45	Actinobacteria, Actinobacteria, Micrococcales, Microbacteriaceae			
CDVBN29 *	YMA	VI	*Clavibacter capsici* LMG 29047^T	99.93	Actinobacteria, Actinobacteria, Micrococcales, Microbacteriaceae			
CDVBN34	TSA	VI	*Clavibacter capsici* LMG 29047^T	-	Actinobacteria, Actinobacteria, Micrococcales, Microbacteriaceae			
CDVBN89*	869 1/10	VII	*Microbacterium yannicii* DSM 23203^T	98.95	Actinobacteria, Actinobacteria, Micrococcales, Microbacteriaceae			

Table 1. *Cont.*

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
CDVBN50 *	869 1/10	VIII	*Microbacterium yannicii* G72^T	100	Actinobacteria, Actinobacteria, Micrococcales, Microbacteriaceae			
CDVBN46A	869 1/10	IX	*Arthrobacter humícola* KV-653^T	-	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN60 *	869 1/10	IX	*Arthrobacter humícola* KV-653^T	99.71	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN84 *	TSA	X	*Arthrobacter pascens* DSM 20545^T	98.73	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
PDABN28 *	869 1/10	XI	*Micrococcus yunnanensis* YIM 65004^T	99.57	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN49 *	869 1/10	XII	*Pseudarthrobacter oxydans* ATCC 14358^T	99.58	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN42 *	869 1/10	XIII	*Pseudarthrobacter oxydans* ATCC 14358^T	99.58	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN43	869 1/10	XIII	*Pseudarthrobacter oxydans* ATCC 14358^T	-	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			

Table 1. *Cont.*

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
CDVBN44	869 1/10	XIII	*Pseudarthrobacter oxydans* ATCC 14358^{T}	-	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN53 *	869 1/10	XIV	*Pseudarthrobacter oxydans* ATCC 14358^{T}	99.58	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN73	869 1/10	XIV	*Pseudarthrobacter oxydans* ATCC 14358^{T}	-	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN57 *	869 1/10	XV	*Pseudarthrobacter oxydans* ATCC 14358^{T}	99.58	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN61	869 1/10	XV	*Pseudarthrobacter oxydans* ATCC 14358^{T}	-	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN51 *	869 1/10	XVI	*Pseudarthrobacter oxydans* ATCC 14358^{T}	99.58	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN33 *	TSA	XVII	*Pseudarthrobacter siccitolerans* LMG 27359^{T}	99.44	Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae			
CDVBN72 *	869 1/10	XVIII	*Nocardioides cavernae* YIM A1136^{T}	99.36	Actinobacteria, Actinobacteria, Propionibacteriales, Nocardioidaceae			

Table 1. *Cont.*

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
CDVBN90 *	869 1/10	XIX	*Nocardioides cavernae* YIM A1136^{T}	99.36	Actinobacteria, Actinobacteria, Propionibacteriales, Nocardioidaceae			
CDVBN101	869 1/10	XIX	*Nocardioides cavernae* YIM A1136^{T}	-	Actinobacteria, Actinobacteria, Propionibacteriales, Nocardioidaceae			
CDVBN102 *	869 1/10	XX	*Micromonospora coxensis* DSM 45161^{T}	99.86	Actinobacteria; Actinobacteria; Micromonosporales; Micromonosporaceae			
PDABN18 *	869 1/10	XXI	*Flavobacterium pectinovorum* DSM6368^{T}	99.09	Bacteroidetes, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae			
PDABN27 *	869	XXII	*Staphylococcus cohnii* subsp. *cohnii* ATCC 29974^{T}	100	Firmicutes, Bacilli, Bacillales, Staphylococcaceae			
CDVBN19 *	869	XXIII	*Staphylococcus cohnii* subsp. *cohnii* ATCC 29974^{T}	99.93	Firmicutes, Bacilli, Bacillales, Staphylococcaceae			
CDVBN54	869 1/10	XXIV	*Bacillus aryabhattai* JCM 13839^{T}	-	Firmicutes, Bacilli, Bacillales, Bacillaceae			
CDVBN55	869 1/10	XXIV	*Bacillus aryabhattai* JCM 13839^{T}	-	Firmicutes, Bacilli, Bacillales, Bacillaceae			
CDVBN58	869 1/10	XXIV	*Bacillus aryabhattai* JCM 13839^{T}	-	Firmicutes, Bacilli, Bacillales, Bacillaceae			
CDVBN68 *	YMA	XXIV	*Bacillus aryabhattai* JCM 13839^{T}	99.86	Firmicutes, Bacilli, Bacillales, Bacillaceae			

Table 1. *Cont.*

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
CDVBN9 *	869 1/10	XXV	*Bacillus megaterium* NBRC 15308^{T}	100	Firmicutes, Bacilli, Bacillales, Bacillaceae			
CDVBN91 *	869 1/10	XXVI	*Bacillus niacini* IFO 15566^{T}	99.38	Firmicutes, Bacilli, Bacillales, Bacillaceae			
PDABN29 *	869 1/10	XXVII	*Bacillus safensis* FO-36B^{T}	99.93	Firmicutes, Bacilli, Bacillales, Bacillaceae			
PDABN11	TSA	XXVIII	*Bacillus siamensis* PD-A10^{T}	-	Firmicutes, Bacilli, Bacillales, Bacillaceae			
PDABN19B *	TSA	XXVIII	*Bacillus siamensis* PD-A10^{T}	99.86	Firmicutes, Bacilli, Bacillales, Bacillaceae			
CDVBN6 *	869	III	*Bacillus simplex* LMG 25856^{T}	99.93	Firmicutes, Bacilli, Bacillales, Bacillaceae			
CDVBN18 *	869	XXIX	*Pseudomonas baetica* A390^{T}	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN66 *	YMA	XXX	*Pseudomonas baetica* A390^{T}	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN28 *	YMA	XXXI	*Pseudomonas baetica* A390^{T}	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN2	YMA	XXXII	*Pseudomonas baetica* A390^{T}	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN4 *	YMA	XXXII	*Pseudomonas baetica* A390^{T}	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			

Table 1. *Cont.*

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
CDVBN8 *	869	XXXIII	*Pseudomonas baetica* A390^T	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN41 *	869 1/10	XXXIV	*Pseudomonas baetica* A390^T	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN45	869 1/10	XXXIV	*Pseudomonas baetica* A390^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN38 *	869 1/10	XXXV	*Pseudomonas baetica* A390^T	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN39	869 1/10	XXXV	*Pseudomonas baetica* A390^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN37	869 1/10	XXXV	*Pseudomonas baetica* A390^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN22 *	YMA	XXXVI	*Pseudomonas baetica* A390^T	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			

Table 1. *Cont.*

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
CDVBN23 *	YMA	XXXVII	*Pseudomonas baetica* A390^T	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN70	YMA	XXXVIII	*Pseudomonas baetica* A390^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN71 *	YMA	XXXVIII	*Pseudomonas baetica* A390^T	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN13 *	TSA	XXXIX	*Pseudomonas brassicacearum* subsp. *brassicacearum* DBK11^T	99.72	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN14	TSA	XXXIX	*Pseudomonas brassicacearum* subsp. *brassicacearum* DBK11^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN62 *	YMA	XL	*Pseudomonas brassicacearum* subsp. *brassicacearum* DBK11^T	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN47 *	869 1/10	XLI	*Pseudomonas brassicacearum* subsp. *brassicacearum* DBK11^T	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN25 *	YMA	XLII	*Pseudomonas brassicacearum* subsp. *brassicacearum* DBK11^T	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			

Table 1. *Cont.*

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
CDVBN27	YMA	XLII	*Pseudomonas brassicacearum* subsp. *brassicacearum* DBK11^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN52 *	869 1/10	XLIII	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* ATCC 49054^T	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN64 *	YMA	XLIV	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* ATCC 49054^T	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN26 *	YMA	XLV	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* ATCC 49054^T	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN108	TSA	XLVI	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* ATCC 49054^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN21 *	TSA	XLVI	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* ATCC 49054^T	99.86	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN10 *	869	XLVII	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* ATCC 49054^T	99.86	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN17	869	XLVII	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* ATCC 49054^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			

Table 1. *Cont.*

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
CDVBN24	YMA	XLVII	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* ATCC 49054^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN11	TSA	XLVII	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* ATCC 49054^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN15	TSA	XLVII	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* ATCC 49054^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN1 *	YMA	XLVIII	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* ATCC 49054^T	99.86	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN69 *	YMA	XLIX	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* ATCC 49054^T	99.86	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN65 *	YMA	L	*Pseudomonas orientalis* CFML96-170^T	99.86	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN3 *	YMA	LI	*Pseudomonas orientalis* CFML96-170^T	99.65	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN20 *	869	LII	*Pseudomonas orientalis* CFML96-170^T	99.79	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			

Table 1. *Cont.*

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
PDABN1 *	TSA	LIII	*Pseudomonas poae* DSM 14936^T	100	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
PDABN14 *	YMA	LIV	*Pseudomonas poae* DSM 14936^T	100	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
PDABN5 *	869	LV	*Pseudomonas thivervalensis* DSM 13194^T	99.86	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
PDABN12	YMA	LV	*Pseudomonas thivervalensis* DSM 13194^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
PDABN3 *	869 1/10	LVI	*Pseudomonas thivervalensis* DSM 13194^T	99.86	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
PDABN4	869	LVI	*Pseudomonas thivervalensis* DSM 13194^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
PDABN6	YMA	LVI	*Pseudomonas thivervalensis* DSM 13194^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
PDABN7	YMA	LVI	*Pseudomonas thivervalensis* DSM 13194^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			

Table 1. *Cont.*

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
PDABN8	YMA	LVI	*Pseudomonas thivervalensis* DSM 13194^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
PDABN13	YMA	LVI	*Pseudomonas thivervalensis* DSM 13194^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
PDABN15	YMA	LVI	*Pseudomonas thivervalensis* DSM 13194^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
PDABN2	YMA	LVI	*Pseudomonas thivervalensis* DSM 13194^T	-	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
CDVBN16 *	869 1/10	LVII	*Pseudomonas thivervalensis* DSM 13194^T	99.86	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae			
PDABN26 *	YMA	LVIII	*Bosea lathyri* DSM 26656^T	99.22	Proteobacteria, Alphaproteobacteria, Rhizobiales, Bradyrhizobiaceae			
CDVBN78 *	869 1/10	LIX	*Devosia psychrophila* Cr7-05^T	99.09	Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae			
CDVBN77 *	869 1/10	LX	*Microvirga aerophila* KACC 12743^T	97.64	Proteobacteria, Alphaproteobacteria, Rhizobiales, Methylobacteriaceae			

Table 1. *Cont.*

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
PDABN20 *	YMA	LXI	*Neorhizobium alkalisoli* CCBAU 01393^T	99.76	Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae			
PDABN21	YMA	LXI	*Neorhizobium alkalisoli* CCBAU 01393^T	-	Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae			
PDABN21B *	869 1/10	LXII	*Agrobacterium nepotum* 39/7^T	100	Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae			
PDABN22B *	869 1/10	LXIII	*Agrobacterium nepotum* 39/7^T	100	Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae			
PDABN19A *	869	LXIV	*Shinella kummerowiae* CCBAU 25048^T	98.53	Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae			
PDABN23 *	869 1/10	LXV	*Shinella kummerowiae* CCBAU 25048^T	98.76	Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae			
PDABN24B	YMA	LXV	*Shinella kummerowiae* CCBAU 25048^T	-	Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae			
PDABN32 *	TSA	LXVI	*Shinella kummerowiae* CCBAU 25048^T	99.76	Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae			
PDABN23A *	TSA	LXVII	*Shinella kummerowiae* CCBAU 25048^T	99.76	Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae			
CDVBN83 *	YMA	LXVIII	*Sphingomonas faeni* DSM 14747^T	99.78	Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae			

Table 1. *Cont.*

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
CDVBN46B *	869 1/10	LXIX	*Massilia suwonensis* 5414S-25^{T}	99.01	Proteobacteria, Betaproteobacteria, Burkholderiales, Oxalobacteraceae			
CDVBN40 *	869 1/10	LXX	*Massilia yuzhufengensis* ZD1-4^{T}	98.59	Proteobacteria, Betaproteobacteria, Burkholderiales, Oxalobacteraceae			
PDABN9 *	YMA	LXXI	*Acidovorax radicis* N35^{T}	99.65	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae			
CDVBN31 *	TSA	LXXII	*Variovorax paradoxus* NBRC 15149^{T}	99.52	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae			
CDVBN59 *	869 1/10	LXXIII	*Herbaspirillum lusitanum* LMG 21710^{T}	100	Proteobacteria, Betaproteobacteria, Burkholderiales, Oxalobacteraceae			
CDVBN63	YMA	LXXIII	*Herbaspirillum lusitanum* LMG 21710^{T}	-	Proteobacteria, Betaproteobacteria, Burkholderiales, Oxalobacteraceae			
CDVBN67	YMA	LXXIII	*Herbaspirillum lusitanum* LMG 21710^{T}	-	Proteobacteria, Betaproteobacteria, Burkholderiales, Oxalobacteraceae			
CDVBN32 *	TSA	LXXIV	*Herbaspirillum lusitanum* LMG 21710^{T}	99.45	Proteobacteria, Betaproteobacteria, Burkholderiales, Oxalobacteraceae			

Table 1. *Cont.*

Strain	Bacterial Growth Medium	879F *	Most Closely Related Type Strain Based on the 16S rRNA Gene	% Similarity with the Most Closely Related Type Strain (16S rRNA)	Taxonomy	Siderophores	Cellulose	P Solub
PDABN25 *	YMA	LXXV	*Shigella flexneri* ATCC 29903^{T}	99.58	Proteobacteria, Gammaproteobacteria, Enterobacterales, Enterobacteriaceae			
CDVBN81 *	TSA	LXXVI	*Acinetobacter johnsonii* ATCC 17909^{T}	99.51	Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae			

Representative strains from each of the 879F groups are marked with asterisks. Grey-highlighted names represent best performing strains regarding the PGP traits. CDV: Castellanos de Villiquera (Salamanca); PDA: Peleas de Arriba (Zamora). Color scale: Grey color means no growth. White color means negative result (growth but no activity). Different shades of blue mean a range from weak (light blue) to strong (dark blue).

Table 2. Results of plant growth-promoting (PGP) tests (IAA-like compounds, solubilization of bi- and tricalcium phosphate, nitrogen fixation, siderophore and cellulose production) performed with strains selected in the plant promotion assay. All the tests were performed in triplicate.

Strain	IAA-like Molecules ($\mu g \cdot mL^{-1}$)	P Solubilization ($Ca_3(PO_4)_2$)	N Fixation	Siderophores	Cellulose	P Solubilization ($CaHPO_4$)
CDVBN4	24.53	+	-	+++	++	+++
CDVBN6	5.34	-	+	+++	++	+++
CDVBN10 *	8.18	+	-	+++	++	+++
CDVBN20 *	75.19	w	-	+++	+	+++
CDVBN21	13.72	+	-	+++	++	+++
CDVBN65	5.14	+	-	+++	-	+++
CDVBN68	10.07	+	-	+++	++	+++
CDVBN69	8.45	+	-	+++	+	+++
CDVBN70	0.00	+	-	+++	++	++

* Selected for further assays. + to +++, positive (range of halo size); -, negative; w, weak.

The results of root and plant height at 5 and 15 days post inoculation (dpi) are shown in Figure 1. The best six bacterial strains according to these results from the 5 dpi samples were re-tested in planta, allowing the seedlings to grow to 15 dpi. All six strains but one significantly increased shoot length compared to the uninoculated control (Figure 1).

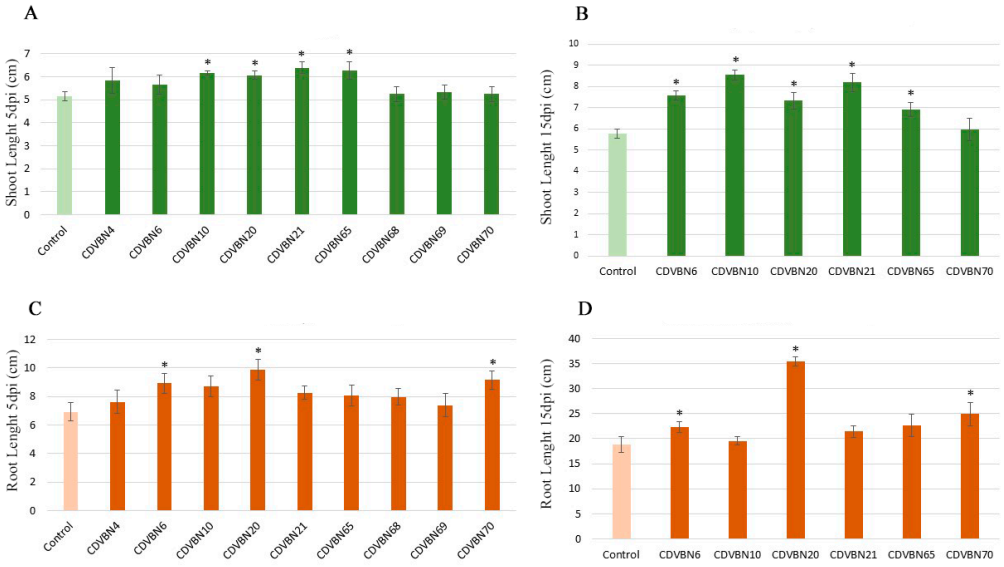

Figure 1. Growth promotion in rapeseed seedlings 5 and 15 days post inoculation (dpi): (**A**) plant height 5 dpi; (**B**) plant height 15 dpi; (**C**) root length 5 dpi; (**D**) root length 15 dpi. Bars indicate the standard error. Histogram bars marked with an asterisk indicate a value significantly different from the negative control ($p = 0.05$) according to Fisher's Protected LSD (Least Significant Differences).

Then, we selected *Pseudomonas brassicacearum* CDVBN10 and *P. orientalis* CDVBN20 to obtain their genome sequence and deepen the in silico study of their PGP capabilities. The reasons for the selection of these two strains are the following: (i) they presented good plant growth-promoting traits according to the in vitro assays, (ii) they presented a capability to promote plant growth at 5 and 15 dpi, and (iii) they belong to the genus *Pseudomonas*, the most abundant genus in plants from both locations, which might be related to a positive role of bacteria of this genus within their host plant (see discussion section).

3.4. Taxonomic Affiliation of the Best Performing Strains

General characteristics of strains CDVBN10 and CDVBN20 genomes are detailed in Table 3, as well as data from Subsystems Categories retrieved from the SeedViewer are shown in Table 4.

Table 3. General genome properties of the PGP strains CDVBN10 and CDVBN20.

Attributes	CDVBN10	CDVBN20
Genome size (bp)	6,180,897	5,666,760
GC Content (%)	60.8	60.6
N50 value	128,213	49,053
L50 value	15	34
Number of contigs (with PEGs)	85	271
Number of subsystems	403	393
Number of coding sequences	5773	5199
Number of RNAs	61	37

Table 4. Number of genes associated with specific functional categories in strains CDVBN10 and CDVBN20.

Number of Genes Related to:	CDVBN10	CDVBN20
Cofactors, vitamins, prosthetic groups, pigments	219	232
Cell wall and capsule	49	49
Virulence, disease and defense	58	60
Potassium metabolism	11	9
Miscellaneous	37	39
Phages, prophages, transposable elements, plasmids	8	3
Membrane transport	194	151
Iron acquisition and metabolism	19	52
RNA metabolism	50	52
Nucleosides and nucleotides	96	101
Protein metabolism	230	212
Motility and Chemotaxis	68	74
Regulation and cell signalling	55	61
Secondary metabolism	4	4
DNA metabolism	101	95
Fatty acids, lipids and isoprenoids	155	138
Nitrogen metabolism	55	19
Dormancy and sporulation	4	1
Respiration	133	111
Stress response	106	102
Metabolism of aromatic compounds	94	71
Amino acids and derivatives	548	481
Sulfur metabolism	24	14
Phosphorus metabolism	35	49
Carbohydrates	316	261

According to the 16S rRNA gene sequence, the most closely related type strains to CDVBN10 are *P. brassicacearum* subsp. *neurantiaca* CIP109457^{T} (99.79%), *Pseudomonas corrugata* DSM7228^{T} (99.65%) and *P. brassicacearum* subsp. *brassicacearum* DBK11^{T} (99.59%). The *gyrB* gene sequence of strain CDVBN10 presented similarities of 94.99%, 92.92%, and 94.71% with those strains, respectively. In the case of the sequence of the *rpoB* gene, the similarities between the strain CDVBN10 and its closest

related species were respectively 97.59%, 95.28%, and 97.00%. Thus, we can conclude that the most closely related type strain of CDVBN10 is *P. brassicacearum* subsp. *neurantiaca* CIP109457^T.

The comparison of the 16S rRNA gene sequence of strain CDVBN20 with the type strains available in databases showed that its most closely related type strains are *P. orientalis* CFML97-170^T (99.66%), *Pseudomonas antarctica* CMS35^T (99.31%), and *Pseudomonas meridiana* CMS38^T (99.25%). In the case of the *gyrB* gene sequence, strain CDVBN20 showed the following similarities with the closest related type strain: 92.48%, 90.73%, and 90.73%, respectively. In the case of the sequence of the *rpoB* gene, the type strains of the most closely related species were not available in the databases. Therefore, according to the 16S rRNA and *gyrB* gene sequences, the most closely related type strain is *P. orientalis* CFML97-170^T.

3.5. Genome in Silico Analysis of Plant Growth-Promoting and Putative Colonization Related Mechanisms

The in silico analyses of the PGP mechanisms of strains CDVBN10 and CDVBN20 showed the presence of genes implicated in several interesting PGP pathways. Both genomes contain genes encoding enzymes involved in the solubilization of inorganic P or in the release of P from other molecules, such as exopolyphosphatases (EC 3.6.1.11), polyphosphate kinases (EC 2.7.4.1), inorganic triphosphatases (EC 3.6.1.25), inorganic pyrophosphatases (EC 3.6.1.1), pyrroloquinoline quinones (PQQ), glucose dehydrogenase PQQ-dependent (EC 1.1.5.2) and gluconate 2-dehydrogenase (EC 1.1.99.3), as well as genes of the Pst system (*pstSCAB*), which is the most conserved member of the Pho regulon [49], and some other genes related to unspecific uptake of this element [24].

Moreover, we found that both bacteria have genes involved in the metabolism of several acids that could solubilize both K and P, such as the genes encoding citrate synthase (EC 2.3.3.1) and malate synthase G (EC 2.3.3.9) responsible for the synthesis of citric acid and malic acid, respectively, genes related with the metabolism of malonic acid (malonate decarboxylase, malonate utilization transcriptional regulator, malonate-semialdehyde dehydrogenase), of gluconic acid (gluconate 2-dehydrogenase (EC 1.1.99.3), of 2-ketogluconic acid (2-ketogluconate kinase (EC 2.7.1.13), 2-ketogluconate transporter) and of lactic acid (D-lactate dehydrogenase, L-lactate dehydrogenase, L-lactate permease). We also found several genes implicated in K transport belonging to the Kup and Kef systems [50].

Regarding iron provision, we found a great number of genes linked with Fe uptake, metabolism and Fe efflux systems, as well as the ones related to the production of pyoverdine, a common siderophore in fluorescent *Pseudomonas* [51]. Regarding IAA, one of the main phytohormones responsible of many plant functions and directly related to plant growth, we found that both genomes have genes encoding for some enzymes related to IAA synthesis, such as the indole-3-glycerol phosphate synthase (EC 4.1.1.48) or the tryptophan synthase (alpha and beta chain; EC 4.2.1.20), amongst others. Nevertheless, we could not find a complete or clear pathway for the biosynthesis of IAA. In addition, using BLASTp search, we found genes encoding 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity in both bacteria, an enzyme which catalyzes the conversion of ACC into ammonia and α-ketobutyrate, avoiding high levels of ethylene synthesis during abiotic stress situations.

Finally, both genomes showed genes involved in lipopolysaccharide (LPS) biosynthesis, such as *ipx*, *waa*, *kdt*, *ept* and *gmh* genes, or genes related to the LPS-assembly, such as *lptD* and *lptE*. Moreover, genes encoding enzymes involved in the synthesis of exopolysaccharides, such as a cyclic β-1,2-glucan synthetase, are in both genomes and *exo* genes, only in the strain CDVBN10. Both genomes also contained genes encoding glycosyl transferases and glycosyl hydrolases, enzymes involved in polysaccharide biosynthesis and biodegradation, and genes encoding transcriptional factors from AraC family.

3.6. Pseudomonas brassicacearum CDVBN10 Displays Beneficial Effects in Rapeseed Plants Cultivated in the Field

According to in vitro, in silico, and in vivo laboratory experiments, *Pseudomonas brassicacearum* CDVBN10 and *P. orientalis* CDVBN20 were shown to be promising plant growth-promoting bacteria. However, and taking into account that *P. brassicacearum* species had been isolated as a root endophyte from several different plants and that the preliminary hypothesis of this study was that bacteria with a good capability to enter plant roots will be more efficient under field conditions, we chose the bacterium *P. brassicacearum* CDVBN10 to tests its capability to promote plant growth in field conditions (a neighbor-joining phylogenetic tree based on the 16S sequence of the strain CDVBN10 and the closest related species of the genus *Pseudomonas* is available in the Supplementary Figure S2). Data from field experiments (Figures 2 and 3) showed a significant increase in both seed weight and shoot biomass in those plants inoculated with *P. brassicacearum* CDVBN10 compared to uninoculated plants. The percentages of the increase in pod number, pod dry weight and shoot dry weight in inoculated plants over the control plants were 216.0%, 174.3%, and 197.8%, respectively. Regarding the nutritional content of the plants, inoculated rapeseed plants present a significantly higher content in N, C and K, whereas uninoculated plants presented higher Fe content than those inoculated with *P. brassicacearum* CDVBN10 (Table 5).

Figure 2. Example of plant growth-promoting effect of *P. brassicacearum* CDVBN10 on *Brassica napus* plant in field experiment; (**A**) control not inoculated, (**B**) plant inoculated with *P. brassicacearum* CDVBN10. Bar represents 12 cm.

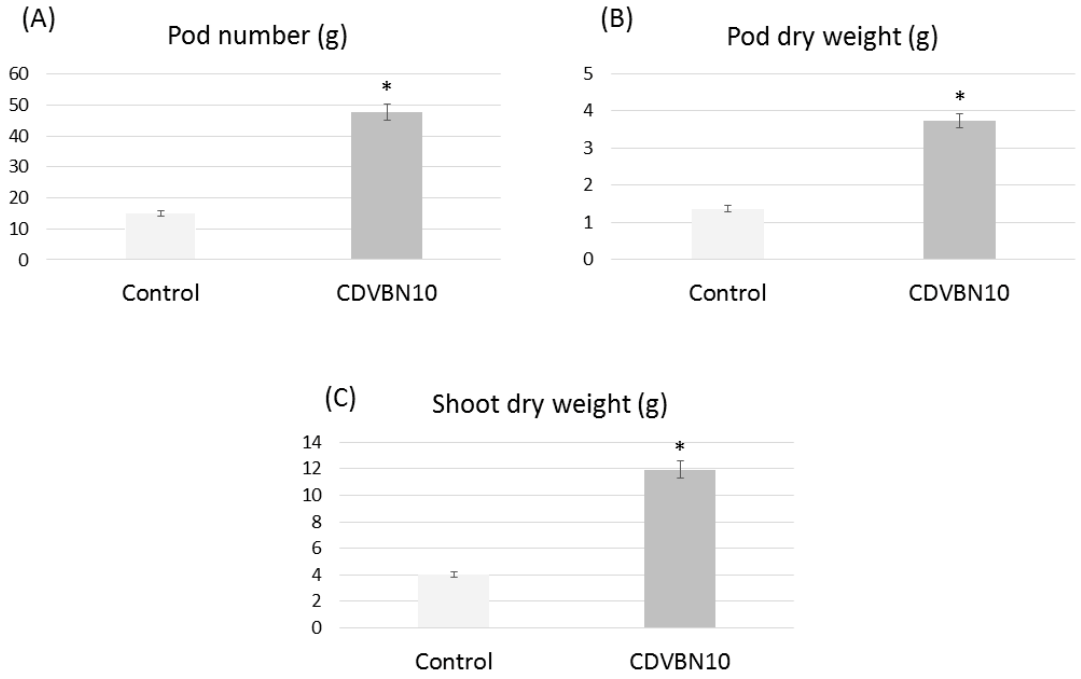

Figure 3. Results of field experiment. (**A**) Pod number, (**B**) pod dry weight (g), (**C**) shoot dry weight (g). Bars indicate the standard error. Histogram bars marked with an asterisk indicate a value significantly different from the negative control ($p = 0.01$) according to Fisher's protected least significant differences (LSD).

Table 5. Effects of *Pseudomonas brassicacearum* CDVBN10 inoculation on nutrient contents of rapeseed plants grown in the field experiment. Values marked with an asterisk indicate a value significantly different from the negative control ($p = 0.05$) according to Fisher's protected least significant differences (LSD).

Treatment	N (g/100g)	C (g/100g)	Fe (mg/kg)	K (g/100g)	P (g/100g)
Control	3.56 ± 0.05	53.69 ± 0.49	67.34 ± 2.52	1.04 ± 0.01	0.58 ± 0.02
CDVBN10	3.82 ± 0.07 *	54.89 ± 0.19 *	59.60 ± 1.50 *	0.99 ± 0.03	0.65 ± 0.03 *

3.7. CDVBN10 Inoculation Does Not Significantly Alter Bacterial Diversity in Rapeseed Roots Grown in the Field Trial

The SMRT PacBio sequencing produced a total of 376,370 reads for the eight samples (four uninoculated and four CDVBN10 inoculated). After the filtering, we obtained a total of 96,105 valid reads (≥ 800 and ≤ 1600 bp), The minimum number of reads per sample was 2381 and the maximum was 21,274. We performed a clustering based on a threshold of 97% similarity and assigned taxonomic rank to generate a total of 3419 OTUs (Table 6). Underrepresented OTUs ($n \leq 2$) were also removed, being a final amount of 2130 OTUs in total.

Setting a level of similarity of 97% as the threshold and removing singletons and doubletons, the average number of OTUs among the samples was 552.2 (±56.81) and 541.2 (±120.73) for CDVBN10-inoculated and uninoculated treatments, respectively. The rarefaction curves for each sample (Figure S1) together with the different alpha diversity indexes (Table 6) show that the most common OTUs are present in the sequencing data. Both alpha (Table 6; Figure 4) and beta diversity (Supplementary Table S1; Figure 5) analyses revealed that there are no statistically significant differences among and within all samples from both treatments and that there are not associations between taxa and treatments (Supplementary Table S2).

Table 6. Number of sequences, OTUs and alpha diversity indexes of bacterial communities present in the 8 samples, 4 from uninoculated and 4 from CDVBN10-inoculated treatments. No significative differences were found ($p > 0.05$).

Samples		Raw Reads	Reads after Processing *	Observed OTUs	PD whole Tree	Chao-1	Shannon	Simpson	Good´s Coverage
CDVBN10	A1	40297	15884	540	69.68	2465.58	5.56	0.75	0.95
	A2	49926	15190	714	71.17	2416.30	8.35	0.97	0.96
	A3	50086	7353	537	44.89	1369.97	7.46	0.97	0.95
	A4	30213	14870	373	53.98	2083.64	3.95	0.61	0.96
Uninoculated	B1	47285	2381	532	31.42	782.39	7.51	0.97	0.90
	B2	61430	4491	525	35.55	1041.56	7.86	0.99	0.93
	B3	44494	14480	501	64.73	2201.01	5.51	0.77	0.95
	B4	52639	21274	648	81.05	2083.64	7.48	0.94	0.96
Total		376370	96105						

* after filtering (< 800 nt > 1300 nt) and chimera removal.

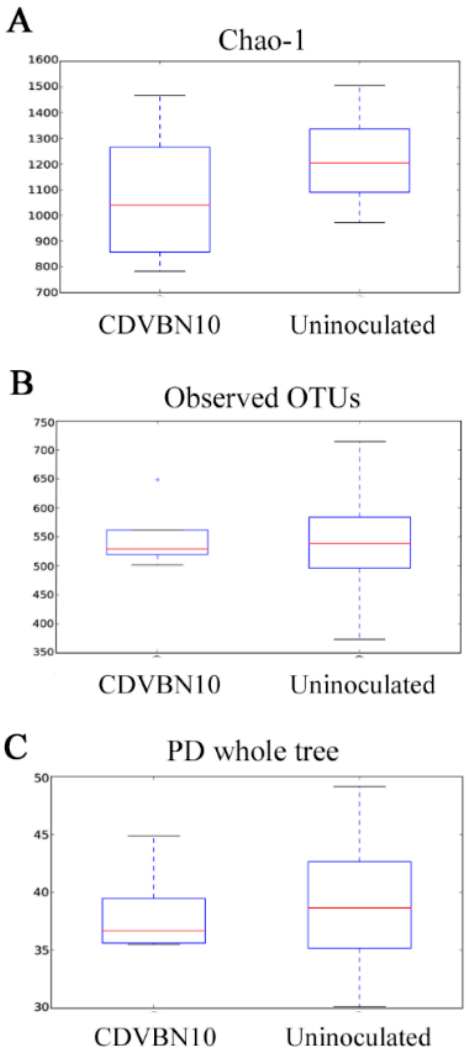

Figure 4. Comparison of alpha diversity between sampling sites; (**A**) boxplots represent Chao-1 index; (**B**) OTU richness/observed OTUs; (**C**) Phylogenetic Diversity (PD) whole tree index. T test was used to detect differences between treatments. No significant differences were found ($p > 0.05$).

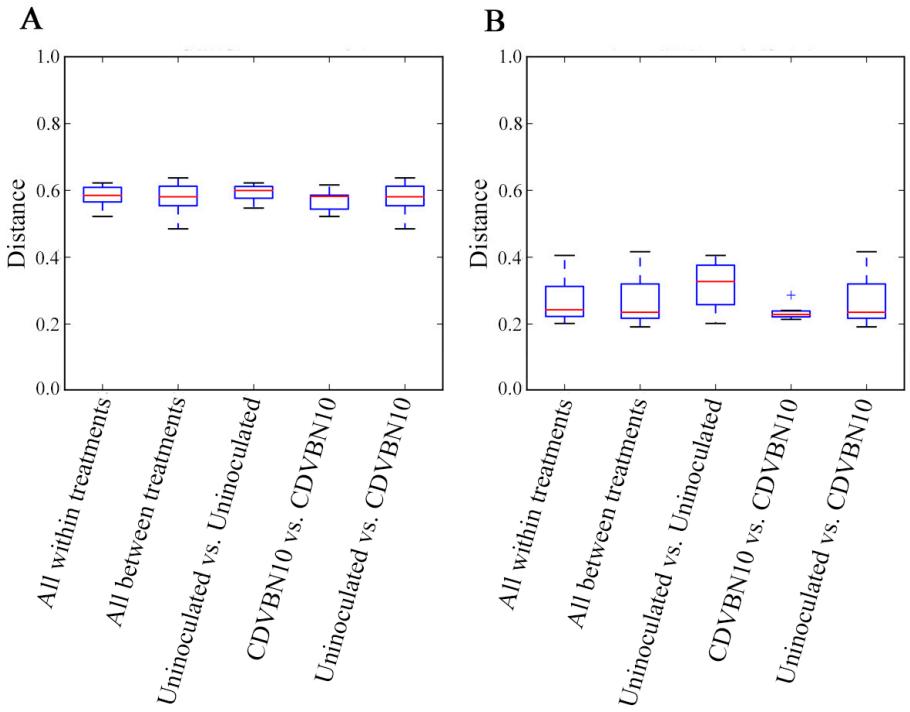

Figure 5. Comparison of beta diversity between sampling sites; (**A**) boxplots represent the unweighted Unifrac distances; (**B**) the weighted Unifrac distances. No significant differences were found among all the samples.

Eleven phyla were identified, with the phylum Proteobacteria, with four of the classes present (Alpha-, Beta-, Gamma- and Deltaproteobacteria), being the phylum with the highest relative abundance (27.8% in uninoculated treatment and 37.1% in CDVBN10-inoculated). The phyla Bacteroidetes (18.0% and 19.0%) and Verrucomicrobia (5.6% and 4.6%) were the second and the third in relative abundance, respectively (Figure 6A). There are more unassigned sequences in the uninoculated (42.6%) than in the CDVBN10-inoculated (34.7%) treatment. The class Betaproteobacteria is the most abundant within both treatments (18.9% and 20.9%), followed by the classes Flavobacteria (9.9% and 11.5%) and Gammaproteobacteria (4.2% and 5.6%) (Figure 6B). The orders Burkholderiales (15.6% and 18.5%), families Commamonadecae (8.8% and 10.6%) and Oxalobacteraceae (6.8% and 7.8%), genera *Polaromonas* (2.1% and 2.6%) and *Janthinobacterium* (2.9% and 3.4%); and Flavobacteriales (9.9% and 11.5%), the family Flavobacteriaceae (9.6% and 11.2%), and the genus *Flavobacterium* (9.6% and 11.2%) are those with the highest relative abundance in both treatments (Figure 6C–E). Other important taxa, such as the order Rhizobiales (2.1%) or the family Pseudomonadaceae (0.7%), showed similar relative abundances in both treatments. Indeed, the genus *Pseudomonas*, which is supposed to be enriched in the CDVBN10-inoculated treatment, showed the same relative abundance (0.7%) in both treatments (Figure 6E).

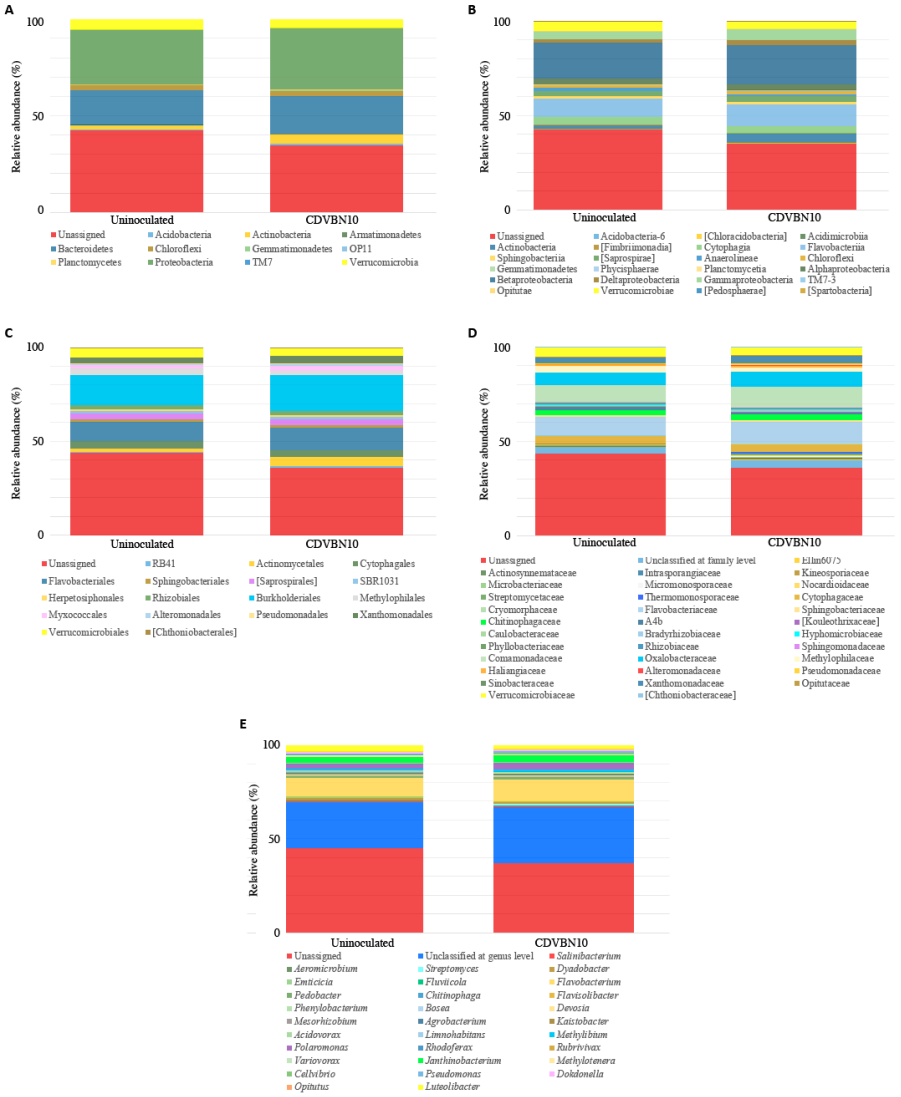

Figure 6. Relative abundance (%) of bacterial taxa found inside roots of rapeseed plants collected in the uninoculated and CDVBN10 inoculated treatments at different taxonomic levels: (**A**) phylum, (**B**) class, (**C**) order, (**D**) family and (**E**) genus. Taxa with relative abundances higher than 0.1% are represented in the charts.

4. Discussion

The results of the present study show a broad biodiversity of bacterial endophytic strains of *B. napus* roots in two soils from Northwest Spain: the 879F-RAPD fingerprinting, which had been proven to be a useful technique to generate different profiles at the intraspecific level in both Gram-positive and negative bacteria [24,26,52], showed the presence of several different profiles among the isolated strains, and the 16S rRNA sequence analysis showed a wide diversity of bacterial species and genera. The dominant genus was *Pseudomonas*, followed by *Pseudoarthrobacter* and *Bacillus*. The genera *Pseudomonas* and *Bacillus* appeared in samples from both localities, while all the other genera were location-specific. Strains from the genera *Pseudomonas*, *Bacillus*, *Rhizobium*, *Staphylococcus*, *Acidovorax*, *Micrococcus*, *Arthrobacter*, *Variovorax*, *Microbacterium*, *Sphingomonas*, *Acinetobacter*, *Devosia* and *Flavobacterium* had already been identified as rapeseed endophytes [19,53–59], while *Micromonospora*, *Massilia*, *Bosea*, *Shinella* and *Agromyces* had been found in soil or rhizosphere associated to *B. napus* roots [57–61]. However, to the best of our knowledge, this is the first report of the association of bacteria from genera *Neorhizobium*, *Microvirga*, *Herbaspirillum*, *Dermacoccus*, *Nocardioides*, *Isoptericola*, *Pseudoarthrobacter*, *Clavibacter* and *Shigella* to *B. napus* plants, although genera such as *Neorhizobium*,

Microvirga and *Herbaspirillum* are well-known PGP bacteria associated to different plants [62–64]. Considering that the plant endosphere is a much more restricted niche than the rhizosphere, these results show a great biodiversity within the isolated strains, probably due to the use of different isolation media.

Regarding the in vitro PGP potential, P is an essential plant nutrient and P deficiency is one of the most important limitations to plant development and crop production, it being estimated that more than 5 billion hectares of land are deficient in P [65]. On the other hand, iron (Fe) is essential for plants, forming part of chlorophyll. Siderophores are molecules that bacteria secrete to solubilize iron, forming a complex ferri-siderophore that can move by diffusion and be returned to the cell or captured by plants [66]. Finally, the production of polysaccharides is an advantage for the strain in order to colonize the plant roots. Amongst those polysaccharides, cellulose is involved in bacterial root colonization and biofilm production—preliminary steps prior to plant growth promotion—and thus, cellulose biosynthesis is important for biofertilizers efficiency [67]. Because of all the mentioned advantages of these PGP bacterial traits, the positive results found for our bacterial isolates suggest the presence of an advantageous endophytic microbiota in rapeseed roots. All isolates except *Nocardioides cavernae* CDVBN101, *Micromonospora coxensis* CDVBN102 and *Bosea lathyri* PDABN26 showed positive results for at least one of the in vitro assayed PGP traits. The best bacteria belonged to the species *Pseudomonas thivervalensis, P. poae, P. baetica, P. brassicacearum, Bacillus aryabhattai* and *Bacillus simplex*. Strains belonging to these species have been previously described as PGP of different plants [68–75].

Thus, we tested the capability of representative bacterial strains from those species to promote rapeseed seedling development. The results from these assays suggest that the strains CDVBN10 and CDVBN20, both belonging to the genus *Pseudomonas*, were the best rapeseed PGPs. The genome analysis of strains CDVBN10 and CDVBN20 showed an interesting genetic PGP potential, as both strains showed positive results in all the PGP traits tested (excepting growth in N-free media). According to the results obtained in the *in vitro* tests performed in this study and the analyses of other genomes of *Pseudomonas* strains [37,76–78], we found a great number of genes linked to Fe uptake, metabolism and efflux systems. In addition, in consonance with the in vitro tests and the results found for other *Pseudomonas* strains [79], both genomes contain gene sequences encoding enzymes that are involved in the solubilization P and K as well as the transport of these elements [20,24,50]. In addition, both bacterial genomes contain genes related to IAA biosynthesis. The lack of detection of a complete IAA biosynthetic pathway may be due to the biases of annotating draft genomes. On the other hand, as with other *Pseudomonas* strains [80], these two bacterial genomes encode the enzyme ACC deaminase; the synthesis of this enzyme would probably confer the plant a better resistance to abiotic stress conditions [81]. The synergy between both IAA synthesis and ACC deaminase activity could lead to a better performance of this plant–bacteria symbiosis [82]. Both bacteria also contain genes related to the biosynthesis of polysaccharides such as a cyclic β-1,2-glucan synthetase [83] in both genomes and *exo* genes [84], only in the strain CDVBN10 and genes encoding glycosyl transferases and glycosyl hydrolases, enzymes involved in polysaccharide biosynthesis and biodegradation [85]; polysaccharides have been proved to play a role in biofilm formation and the colonization of root surfaces [35,86,87]. Both genomes also have genes encoding transcriptional factors from AraC family, which are known as regulators of many processes including the ones involved in the interchange of signals among bacteria [88] and have been revealed as relevant for rhizosphere competition in rhizobia [89].

Strain CDVBN20 belongs to the species *Pseudomonas orientalis*, a bacterium not frequently associated with plant microbiomes, this being, to the best of our knowledge, the first time it has been described as a bacterial species associates to *B. napus*. However, the strain CDVBN10 belongs to the species *Pseudomonas brassicacearum,* which was originally described as a bacterial colonizer of *B. napus* rhizosphere [90]. Moreover, different strains of this species have been isolated as root endophytes from different plants, such as *Salvia miltiorrhiza* Bunge. [69], *Artemisia* sp. [91], *Lavandula dentata* L. [92] and nodules of the legume *Sphaerophysa salsula* (Pall.) DC. [93]. Some studies also reported how this species promotes the growth of *Pisum sativum* L. [94], *Solanum nigrum* L. [95] and *Medicago lupulina* L. [96] plants.

Moreover, the genome sequence analyses of other bacterial strains belonging to this species seem to indicate that this bacterium is a good plant growth promoter and a potential biocontrol agent [97,98]. Considering the results of this study and previous references of the species, we conclude that the strain *P. brassicacearum* CDVBN10 has a good potential as rapeseed biofertilizer and we decided to test its performance under field conditions. The results of our trial, performed with no addition of chemical fertilizers, show a significant increase not only in total plant biomass, but also in seed yields compared to the non-inoculated control plants, confirming that this bacterium has an interesting potential to be employed as a biofertilizer for *Brassica napus* crops, as it has been already for other *Pseudomonas* species inoculated in field trials [99–101], this being, to the best of our knowledge, the first report of a PGP bacterium with potential to specifically promote rapeseed/canola crops which showed an important yield increase in field trials

Interestingly, despite the significant differences in plant development and yields, the analysis of the biodiversity based on amplicon sequencing showed that there are no significant differences in the root bacterial communities of plants inoculated with the strain *Pseudomonas brassicacerarum* CDVBN10 nor in the associated functions of this community. In this sense, our results agree with those of Qiao et al. [102], which showed that the inoculation of a PGPB *Bacillus* strain does not alter the root bacterial microbiome on tomato plants. However, this effect might be strain-specific or context-specific, as suggested by Gadhave et al. [103]; these authors performed several inoculations with different PGPB strains belonging to the genus *Bacillus* and found that there is an infraspecific variation and competition issues within sprouting broccoli roots. The modulation of root microbiomes by addition of biofertilizers based on beneficial strains and other factors is not well-understood and further studies must be performed to elucidate these effects [104].

According to ecological theories [13,105], most bacteria living as root endophytes probably play important roles for the plant development and survival. Thus, in our opinion, the results obtained in this study are very positive: rapeseed plants from the plots inoculated with the strain CDVBN10 showed a clear benefit from the inoculation and their endophytic root microbiome was not altered by the inoculation, so there was not competition of potentially benefiting members of the plant microbiome.

There is an unexpected result in the PacBio data; we were not able to detect any OTU belonging to the phylum Firmicutes. This is a rare event, taking into account that members of this phylum were found within the root bacterial microbiome of *Brassica* plants [106]. However, Lay et al. [61] did not find any Firmicutes in canola roots. Some of the amplicon sequences appeared as unclassified at different taxonomic levels, which might be the reason for lacking some taxa in the amplicon sequencing analyses. These results highlight the importance of combining culturomics and metagenomics for biodiversity studies, because whereas isolated strains can be better identified, amplicon sequences allow us to decipher those members of the community which cannot grow in synthetic conditions or are inhibited by other members of the community in the selected growth conditions of the study.

As the bacterial communities associated to plants, both rhizospheric and endophytic, are strongly influenced by many factors [107–109], further studies on different soils and climate conditions should be performed in order to demonstrate the success of this strain as a biofertilizer for rapeseed crops and the lack of alteration of the root microbiome after its addition; furthermore, the best formulation of the strain to be commercialized as a biofertilizer should also be evaluated.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/11/1788/s1, Data sheet; Supplementary Table S1: Beta diversity results. Statistics corresponding to distance boxplots of Figure 5 (from the main text) according to unweighted and weighted Unifrac distances. Supplementary Table S2: Statistic significance of the relatedness of each OTUs with each treatment group (control samples or CDVBN10 inoculated samples). Supplementary Figure S1: Rarefaction curve for observed bacterial OTUs clustering at 97% 16S rRNA sequence similarity. Curves represent number of observed OTUs from the uninoculated (A1-4) and CDVBN10 inoculated (B1-4) treatments. Supplementary Figure S2: Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strain *P. brassicacearum* CDVBN10 and its closest related type strains. Scale bar = 5 nucleotide (nt) substitutions per 1000 nt.

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