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Response of Soil Bacterial Community and Pepper Plant Growth to Application of *Bacillus thuringiensis* KNU-07

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Abstract: Many *Bacillus* species are among the plant growth-promoting rhizobacteria (PGPR) that promote the growth of many different plant species. This study aimed to investigate the effects of *Bacillus thuringiensis* KNU-07 on the growth of pepper plants and the soil microbiota. We also designed primers specific for the strain KNU-07 to monitor the population in pepper-cultivated soil. Accordingly, a strain-specific primer pair was designed using a database constructed from 16,160 complete bacterial genomes. We employed quantitative PCR (qPCR) to track the abundance of the strain KNU-07 introduced into pepper-cultivated soil using the strain-specific primers. Our study revealed that the strain was found to possess plant growth-promoting (PGP) activities, and it promoted the growth of pepper plants. The soil bacterial community structure due to the application of the PGPR strain was significantly changed after six weeks post-inoculation. In addition, based on qPCR analysis, the population of the introduced strain declined over time. In this study, application of a PGPR strain increased the growth of pepper plants and changed the soil bacterial community structure. The successful results of monitoring of a bacterial strain's population using a single strain-specific primer pair can provide important information about the quantification of bio-inoculants under non-sterile soil conditions.

Keywords: *Bacillus thuringiensis*; *Capsicum annuum*; PGPR; microbiome; strain-specific primer; tracking

1. Introduction

Plant growth-promoting rhizobacteria (PGPR), which are found in the vicinity of crop roots, increase the growth and health of the plants [1]. Considering the growing public concern about the use of chemical fertilizers, there is increasing high demand to use PGPR, such as *Bacillus* spp., *Pseudomonas* spp., *Streptomyces* spp., etc. [2–4]. *Bacillus* is one of the most important genera that provides plants with potent plant growth-promoting effects, and many *Bacillus* species have been successfully used for agricultural purposes as commercial bio-inoculants [5,6]. Some strains of *Bacillus thuringiensis* have been used as a PGPR to improve soil fertility and enhance crop growth [7–9]. In addition, although the effect of PGPR on the indigenous soil bacterial communities and their functional properties has been studied, there is very limited information on the effects of *B. thuringiensis* on bacterial communities

in the soil [10,11]. The beneficial effects of *B. thuringiensis* on plants are due to direct and indirect mechanisms, including nitrogen fixation, siderophore production, plant nutrient solubilization, and plant growth hormone production [12–14]. However, the plant responses are often variable due to inconsistent performance of inoculants under field conditions [15].

The ability of inoculants to survive in the soil is an important factor for their ability to function under field conditions [15,16]. Hence, quantification of inoculants in the soil is helpful to determine the success of PGPR under field conditions [1,6,17]. However, measuring the spatiotemporal dynamics of PGPR in the environment remains challenging [18,19]. Various culture-dependent and culture-independent methods have been employed to track and quantify bio-inoculants in the soil [20,21]. However, culture-dependent methods are only successful under sterile conditions, and less than 1% of the soil microbial diversity is recovered with such methods [21,22]. On the other hand, culture-independent methods, such as reporter nucleic acid-based, gene-based, and immunological methods, are capable of detecting less abundant, slow-growing, and unculturable bacteria [8–10]. However, most culture-independent methods are incapable of monitoring population dynamics at a species level, making it difficult to determine the fate of some strains [23,24].

In this study, we investigated the effects of the PGPR strain KNU-07 (hereafter referred to as KNU-07) on the growth of pepper plants and soil bacterial community composition. More importantly, we developed a strain-specific primer pair and developed a qPCR protocol to track the quantity of *Bacillus thuringiensis* KNU-07 in pepper-cultivated soil in a greenhouse. KNU-07 increased the growth of pepper plants, and the application of KNU-07 significantly changed the soil bacterial community structure after six weeks. The established strain-specific primer was successful in quantifying and monitoring KNU-07 in non-sterile soil conditions.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

The genome of the rhizospheric bacterial strain used in this study, KNU-07, was assembled using PacBio RSII and comprised 6,152,737 bp. KNU-07 was cultured in Luria-Bertani (LB) broth and LB agar (Difco Laboratories, Sparks, MD) and incubated for 24 h at 30 °C. Bacterial strains used for in vitro PCR are indicated in Table S1.

2.2. Bioinformatics Approach for Designing a Strain-Specific Primer for KNU-07

2.2.1. Designing a Primer to Target a Unique Sequence of KNU-07

Strain-specific primers were designed using a Python script that was developed in house. The complete genome sequence of KNU-07 was cut into truncated fragments of 500 bp. BLASTN was used to search for each fragment in a custom database constructed by downloading 16,160 complete bacterial genomes from the NCBI Genome Browse section (updated on 27 December 2019). The BLASTN searches were performed with the following parameters: ungapped alignment (-ungapped), no filter query sequence with dust (-dust no), and apply filtering locations as soft masks (-soft_masking false). Unique fragments containing regions that had no overlap with any complete genome in the NCBI custom database were chosen for further analysis. A primer pair targeting the unique sequence was designed using the web-based tool Primer-BLAST (NCBI Primer-BLAST).

To validate the selected unique fragment, BLASTN was used as mentioned above. The primer pair was checked to ensure homology to the KNU-07 unique sequence region, and the specificity of the primer was validated by performing in silico PCR evaluation using ecoPCR software on the NCBI bacteria complete genome database [25]. The number of mismatches in the binding regions of the target sequence for either forward or reward primers were set to 0 to 2. The targeted PCR product size was set to a minimum of 50 bp and a maximum of 500 bp. For comparison, a universal primer pair, 27F/1492R, which amplifies a region of the 16S rRNA gene in prokaryotes, was used as a positive

control (Table 1). Finally, an ecotaxstat script was used to summarize taxonomy information from the in silico PCR products.

Table 1. KNU-07-specific primers and universal primers used in this study.

Primer Name	Primer Sequence (5′→3′)	Reference
Strain-specific primers		
KNU07F	TGCTCTTTCTGGATTATTCCTTGAG	This study
KNU07R	CATCCTTTTGTAGAAGGTATTGCCA	This study
Universal primers		
27F	AGAGTTTGATCMTGGCTCAG	Lane, 1991
1492R	TACGGYTACCTTGTTACGACTT	Lane, 1991
515F	GTGCCAGCMGCCGCGG	Lane, 1991
907R	CCGTCAATTCMTTTRAGTTT	Lane, 1991

2.2.2. In Vitro Validation of the Strain-Specific Primer Pair

PCR was performed to verify whether the primer pair designed by in silico PCR analysis amplified the unique sequence of KNU-07. In addition, we conducted additional PCR assays to investigate whether the strain-specific primer pair amplified genomic DNA from other bacterial species and environmental samples in vitro (Tables S1 and S2). Each PCR reaction contained 10 ng of template DNA, 0.2 µL of each primer (10 µM), 5 µL of EmeraldAmp GT PCR Master Mixture (Takara Korea Biomedical Inc., Seoul, Korea), and sterile distilled water to a total volume of 20 µL. PCR amplifications were carried out using the following cyclic program: initial denaturation at 95 °C for 7 min, followed by 30 cycles of 30 s denaturation at 95 °C, 55 °C annealing for 30 s, 72 °C extension for 30 s, and a final extension at 72 °C for 5 min.

2.3. In Vitro Plant Growth-Promoting (PGP) Traits Assay

An in vitro assay was carried out to evaluate the effect of KNU-07 on plant growth potential. The indole acetic acid (IAA) production potential was evaluated following the method of Gordon and Weber [26]. The concentration of IAA was quantified based on a standard curve of pure IAA (Sigma-Aldrich, St. Louis, MO, USA). IAA identity and its purity were confirmed by gas chromatography–mass spectrometry with a SIM (6890N network GC system, and 5973 network mass selective detector; Agilent, CA, USA). In addition, the potential of the strain to exhibit urease activity [27], siderophore production [28], and phosphate solubilization [29] was determined.

2.4. Greenhouse Experiment

2.4.1. Plant Material and Bacterial Strain Preparation

Seeds of hot pepper (*Capsicum annuum* cv. CM334) were used in this experiment. KNU-07 was incubated at 30 °C for 24 h at 200 rpm. The pellet was collected after centrifugation, washed, and resuspended in sterile distilled water. The bacterial inoculum was adjusted using a sterile distilled water to concentrations of 7.8×10^6 cells mL^{-1} soil and 7.8×10^8 cells mL^{-1}.

2.4.2. In Vivo Assay

The effect of strain KNU-07 on the growth of pepper plants in pots under greenhouse conditions for two months was assessed. Pepper seeds were surface-sterilized with ethanol (70%) for 1 min and soaked in a disinfectant solution (Clorox, distilled water, and 0.05% Triton X-100 in a 3:2:2 ratio (v/v/v)) for 5 min and washed 7–10 times with sterile, deionized, distilled water. Pepper seeds were vernalized for 48 h in a refrigerator at 4 °C and germinated by placing the seeds on sterile, wet filter paper in a growth chamber for 7 days at 30 °C. The germinated seeds were then sown in plastic trays containing mixed soil. The mixed soil was composed of garden soil and Biosangto-Mix soil (Heung Nong Co.,

Ltd., Pyeongtaek, Republic of Korea) in a 1:9 ratio (v/v). The pepper seedlings were incubated in a growth chamber (25 °C, 65% relative humidity, and cycles of 16 h light and 8 h dark). After two weeks, uniform-sized pepper seedlings having shoots approximately 5 cm in height were each transplanted into a pot containing 300 g of mixed soil. To assess the effect of KNU-07, the soil of some pots was inoculated with 3.85 mL of KNU-07 at one of the following concentrations: 1.0×10^5 cells g^{-1} soil and 1.0×10^7 cells g^{-1} soil. Application of bio-inoculants at 1.0×10^5 cells g^{-1} soil is a very common practice in South Korea. Seedlings treated only with sterile distilled water served as the non-inoculated control. The experiment was replicated three times with five plants per replication. After 11 weeks of treatment, plant growth data, including plant shoot length, root length, and total biomass, were recorded.

2.4.3. DNA Extraction from Pure Cultures and Soil Samples

To analyze the soil bacterial community, the soil where *Capsicum annuum* cv. CM334 was growing in each pot was sampled weekly. Very small amounts of soil sample (less than one gram) were taken at five different sites in each pot and pooled into a composite sample per pot. Genomic DNA from the soil and the strain culture was extracted using a Power Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. DNA concentrations were determined using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). For strain-specific PCR assays, KNU-07 genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA).

2.4.4. DNA Library Preparation and Amplicon Sequencing

The diversity of the soil bacterial community was assessed by amplifying and analyzing the V4-V5 hypervariable region of 16S rRNA gene using the universal primer pair 515F/ 907R (Table 1). The V4-V5 primer pair was tailored with Ion Torrent PGM adapter and barcode sequences, which are unique to each sample. The PCR reaction (50 µL) contained 1 ng template DNA, 1 µL of each primer, and 25 µL of EmeraldAmp GT PCR Master Mixture (Takara, Japan). The PCR conditions were as follows: initial denaturation at 95 °C for 7 min; 10 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; 30 cycles of denaturation at 95 °C for 30 s, annealing and extension at 72 °C for 45 s; and a final extension at 72 °C for 5 min.

Ion Torrent PGM sequencing technology and data analysis were used to sequence the amplified products. The quality of the amplified DNA library was assessed using an Agilent 2100 Bioanalyzer with a High-Sensitivity DNA (HS DNA) Kit (Agilent Technology, Santa Clara, CA, USA). The amplified DNA library was diluted to 6 pM to perform emulsion PCR with Ion Sphere Particles (ISPs) in the Ion OneTouch System II (Thermo Fisher Scientific), followed by enrichment for template-positive Ion Sphere Particles using Dynabeads MyOne Streptavidin C1 beads (Thermo Fisher Scientific, Waltham, MA, USA). Each sample was loaded on barcoded chips (Ion 316 Chip Kit v2 BC). Sequencing was carried out using the Ion Torrent PGM system and an Ion PGM Hi-Q Sequencing Kit (Thermo Fisher Scientific). The Torrent Suite Software, along with Ion Torrent PGM specific pipeline software, was employed to generate sequence reads, trim adapter sequences, filter, and exclude poor signal profile reads. Quality-filtered sequence reads were analyzed using a QIIME package (V1.9.1). Operational taxonomic units (OTUs) having 97% similarity were selected by an average neighbor algorithm and were identified using the sequence database of the National Center for Biotechnology Information (NCBI).

2.4.5. Continuous Tracking of KNU-07 Using qPCR

The abundance of inoculated KNU-07 in pepper-cultivated soil was monitored using qPCR with strain-specific primers (Table 1). The total bacteria in the soil were quantified using qPCR with the universal primer pair (27F/1492R) targeting a 16S rRNA gene (Table 1). Each PCR reaction mixture (10 µL) consisted of 10 ng of DNA from the soil sample, 0.3 µL of each KNU07 specific primer (10 µM), and MG 2X qPCR mix (SYBR green, MGmed). qPCR reactions were performed in triplicate under the following cycling conditions: 95 °C for 15 min, followed by 35 cycles of denaturing at 95 °C for 30 s,

annealing at 55 °C for 30 s and extension at 72 °C for 30 s. Gel electrophoresis using a CFX Real-Time PCR Detection System (BioRad) was conducted to ensure the appropriate size of the amplified products.

A standard curve based on copy number was used to determine the abundance of KNU-07 and total bacteria in the soil. Briefly, a six-fold serial dilution of amplicons of KNU-07 unique sequence was prepared in triplicate. The copy number of each concentration was calculated based on the amplicon concentration and length. A regression equation was calculated based on the cycle threshold (Ct) value to the known amount of serially diluted copy number of the unique sequence. By using the standard curve, the abundance of KNU-07 was deduced and expressed as the number of genome equivalents. A genome equivalent corresponds to the number of KNU-07 cells. In addition, the abundance of the total bacteria was determined using qPCR with a universal primer pair that amplifies a conserved region of the 16S rRNA genes of multiple bacteria species.

2.5. Nucleotide Accession Numbers

The complete genome sequence of *B. thuringiensis* KNU-07 was deposited in GenBank under accession number CP016588. The unique DNA sequence of KNU-07, which was used to design the strain-specific primers, is located at the sequence position 1,904,488 bp to 1,904,728 bp. The NGS data of all raw sequence reads were deposited in the NCBI Short Read Archive (SRA) database under accession number SRP243872.

2.6. Statistical Analysis

The alpha diversity of KNU-07-treated and control samples was analyzed using taxonomic diversity indices, such as the Shannon index, Simpson's index, and the number of observed OTUs. The community diversity difference was analyzed based on principal coordinate analysis (PCoA) using Bray–Curtis distances in QIIME1. A dissimilarity analysis of Bray–Curtis based on permutational multivariate analysis of variance (PERMANOVA, ADONIS function) [30] and an analysis of similarity (ANOSIM function) [31] were conducted to determine the impact of KNU-07 application on the soil bacterial community composition. The abundance of predicted gene function of the soil bacterial community in each experimental sample was determined by the PICRUSt pipeline using an OTU table normalized to the 16S rRNA gene copy number [32]. The data of predicted function were analyzed using the STAMP software package [33]. All data of greenhouse experiments were arranged in a randomized design with at least three replications. Analysis of variance (ANOVA) was performed for plant growth parameters using SAS software version 9.4 [34], and treatment means were separated using post hoc Tukey significant difference (HSD) tests.

3. Results

3.1. In Silico and in Vitro PCR Verification of KNU-07-Specific Primer Pairs

The genome of KNU-07 was truncated into 500 bp fragments, and 10,687 fragments were found. Among these, 81 unique windows were identified, and one window (located at 1,904,488 bp to 1,904,728 bp) was selected for designing the primers. A primer targeting a unique sequence of KNU-07 was designed to have 25 bp (Table 1) using the Primer-BLAST tool on the NCBI web site.

For the in silico PCR analysis, complete genomes of 16,160 bacteria comprising 52 phyla, 173 orders, 1130 genera, and 3747 species were used. A universal primer pair, 27F/1492R, targeting the bacterial 16S rRNA gene matched perfectly with 76% of the species tested (no mismatch), 89% of species had one mismatch, and 92% of species had two mismatches. On the other hand, our strain-specific primer pair targeting the unique sequence of KNU-07 had a perfect match to only one genome, that of *B. thuringiensis* KNU-07 (Table 2). Even by increasing the number of mismatches, no bacterial species other than KNU-07 was found to match, indicating that the primer pair was highly specific to KNU-07.

Table 2. In silico PCR verification that strain-specific primers target sequences unique to KNU07.

Category	Primer	Taxonomic Level	Total Taxa	Number of Mismatches per Primer		
				Perfect Match	1 Mismatch	2 Mismatches
Bacterial 16S rRNA gene	27F/1492R	Phylum	52	26 (50%)	35 (67%)	41 (79%)
		Class	81	48(59%)	63 (78%)	70 (86%)
		Order	173	114 (66%)	140 (81%)	148 (86%)
		Family	367	262 (71%)	313 (85%)	323 (88%)
		Genus	1130	830 (73%)	993 (88%)	1019 (90%)
		Species	3747	2843 (76%)	3331 (89%)	3453 (92%)
KNU-07 unique region	KNU07F/KNU07R	Phylum	52	1*	1*	1*
		Class	81	1*	1*	1*
		Order	173	1*	1*	1*
		Family	367	1*	1*	1*
		Genus	1130	1*	1*	1*
		Species	3747	1*	1*	1*

* Perfect matches with *B. thuringiensis* KNU-07.

To verify whether our designed primer pair specifically detected KNU-07, an in vitro PCR assay was conducted using DNA samples from pure cultures of 28 bacterial strains. The results showed that the primer pair amplified the expected band size of 241 bp from strain KNU-07; however, no visible band was detected with any other bacterial strain, including *Bacillus* spp., other than KNU-07 (Figure S1). Our strain-specific primer can precisely detect and distinguish KNU-07 from other tested *Bacillus* species. Furthermore, the discrimination power of the KNU-07 strain-specific primer was verified using 28 diverse environmental DNA samples. The results confirmed that the strain-specific primer was able to successfully amplify KNU-07 with the expected band size of 241 bp, while no visible band was detected in any environmental sample (Figure S1), demonstrating that the strain-specific primer was selective in detecting KNU-07.

3.2. PGP Activity of KNU-07

KNU-07 was positive for in vitro PGP activities, including IAA production, siderophore production, phosphate solubilization, and urease activities (Figure S2). In addition, gas chromatography/mass spectrometry experiments revealed that the amount of IAA produced by KNU-07 with and without a tryptophan supplement was 4.886 and 0.167 μg mL^{-1}, respectively. The in vivo effects of KNU-07 inoculated at different concentrations on the growth of pepper plants was determined under non-sterile conditions. After 11 weeks of growth, a significant ($p < 0.05$) difference was found between bacterized and non-inoculated pepper seedlings (Figure 1). Plants treated with high concentrations of KNU-07 exhibited significant increases in root length (30.7%), shoot length (19.7%), and total dry biomass (30.7%) compared to non-inoculated control plants (Figure 1).

3.3. Response of the Soil Bacterial Community to KNU-07

The effect of KNU-07 on the composition of the soil bacterial community in pepper-cultivated soil was analyzed by the Ion Torrent PGM platform based on 16S rRNA gene amplicon sequences. In this study, 3146 observed OTUs, 27 phyla, and 408 genera were identified by a BLASTN search against the Green gene database (data not shown). The results revealed that the alpha diversity indices, the Shannon index, and the number of observed OTU increased similarly over time in inoculated and non-inoculated control samples (Figure 2A). The Simpson's index showed that diversity increased with each treatment, except when KNU-07 was applied at the highest concentration (1.0×10^7 cells g^{-1} soil) (Figure 2B). During the first two weeks, Simpson's index was low in soil treated with the highest concentration of KNU-07. However, after three weeks, Simpson's index increased over time (Figure 2C).

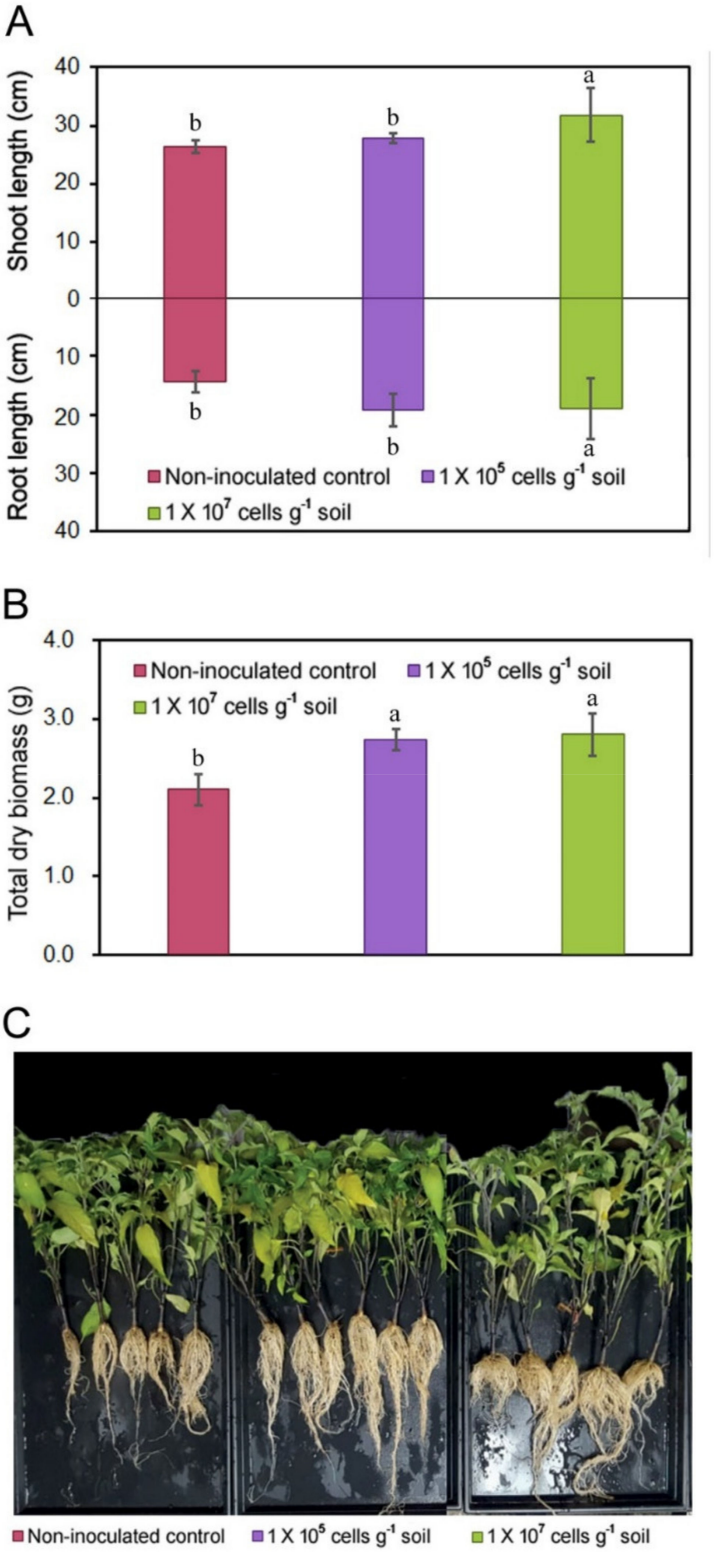

Figure 1. Effect of KNU-07 inoculation on the growth of pepper plants over 11 weeks post-inoculation in greenhouse conditions. The numerical value of (**A**) root length and shoot length and (**B**) total dry biomass. (**C**) Pictorial view of pepper plants inoculated with the indicated concentrations of *B. thuringiensis* KNU-07. Non-inoculated plants served as control. Mean values having different letters in each of the growth parameters are significantly different ($p \leq 0.05$).

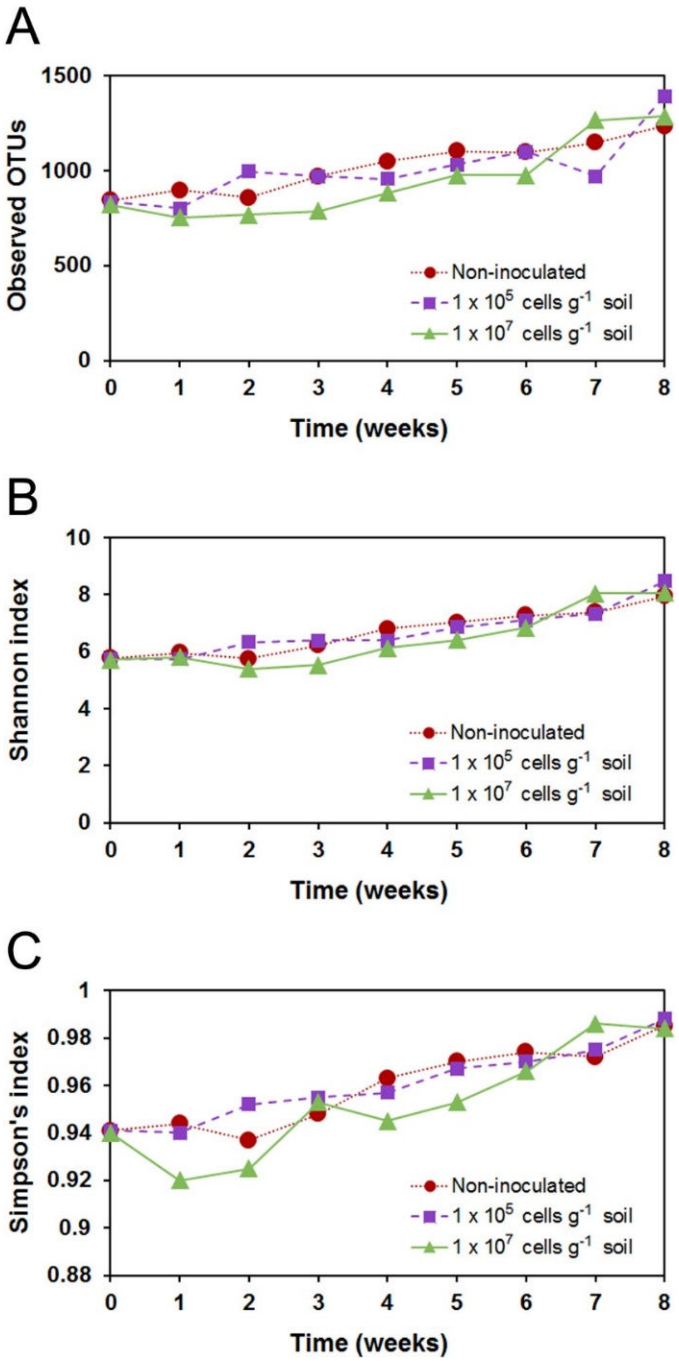

Figure 2. Taxonomic α-diversity analysis: (**A**) observed operational taxonomy units (OTUs); (**B**) Shannon index; (**C**) Simpson's index. Non-inoculated control was not inoculated with *B. thuringiensis* KNU-07.

Xanthomonadales and Saprospirales were the two most abundant orders in this study regardless of the KNU-07 application, and the abundance of these orders gradually decreased over time (Figure 3). In contrast, the abundance of orders Rhizobiales and Ellin329 increased over time in all samples, including controls. The abundance of Acidobacteriales decreased over time in all samples. The abundance of Bacillales, the order to which KNU-07 belongs, was comparatively high in KNU-07-inoculated soil during the first three weeks, but then it decreased (Figure 3). At genera level, the abundance of *Bacillus* spp. was comparatively high in soil inoculated with a high concentration of KNU-07 (1.0×10^7 cells g^{-1} soil) (Figure 4). At a higher concentration of KNU-07, although the

abundance of *Bacillus* spp. was decreasing over time, the abundance of *Bacillus* spp. was still higher than the remaining treatments. The abundance of *Bacillus* spp. in the soil inoculated with a lower concentration of KNU-07 (1.0×10^5 cells g^{-1} soil) and non-inoculated control was comparatively higher in the last three weeks (Figure 4). However, it is important to note that the resolution power of NGS of 16S rRNA coding region is not strong enough to discriminate KNU-07 from indigenous *Bacillus* spp. Hence, we designed a strain-specific primer for KNU-07 to monitor the population dynamics of KNU-07 using qPCR with strain-specific primers.

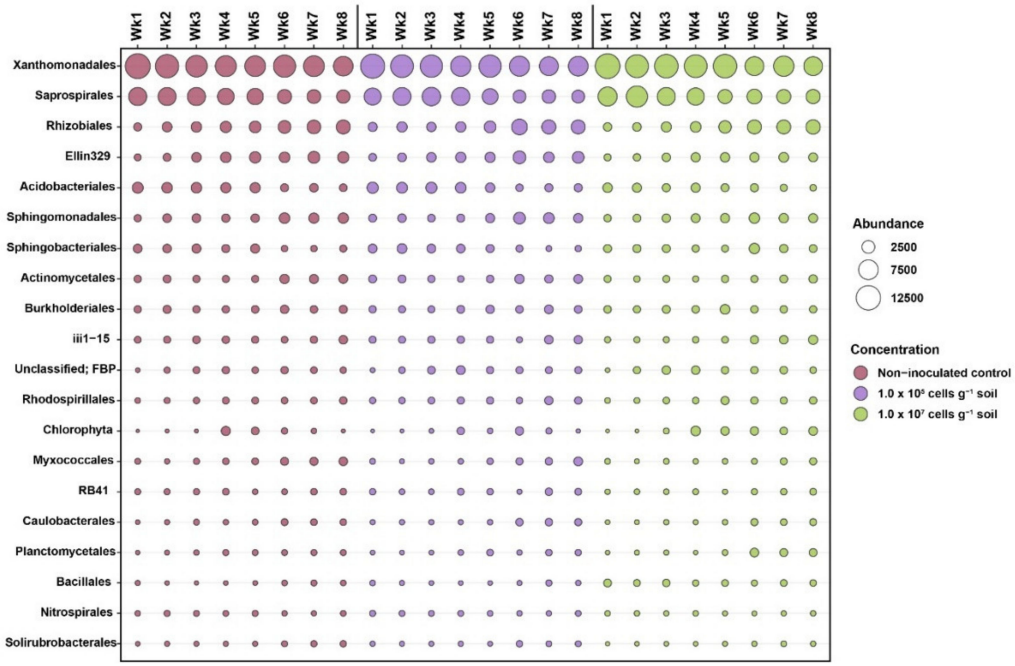

Figure 3. Bubble plot showing the abundance of the bacterial community at an order level based on the 16S rRNA gene in pepper-cultivated soil inoculated with strain KNU-07.

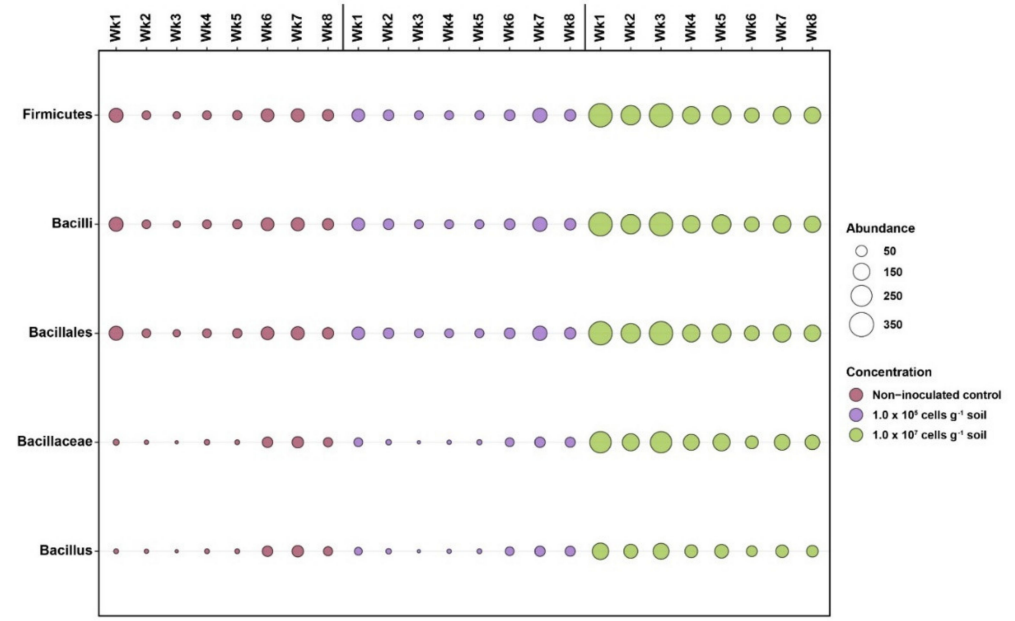

Figure 4. Bubble plot showing the abundance of bacterial taxa, where KNU-7 belongs, over eight weeks post-inoculation using 16S rRNA gene sequencing.

The results of the beta diversity analysis based on principal coordinate analysis (PCoA) at an OTU level revealed that the soil bacterial community compositions were separated over time in all treatments including control (Figure 5). More importantly, bacterial community compositions of the soil treated with a high concentration of KNU-07 were separated from non-inoculated control samples in the last three weeks (Figure 5). These test results were similar to non-parametric statistical analyses based on ADONIS and ANOSIM. The analysis confirmed that the beta diversity between KNU-07-bacterized and non-inoculated control samples was significantly ($p < 0.05$) separated six weeks post-inoculation (Table S3).

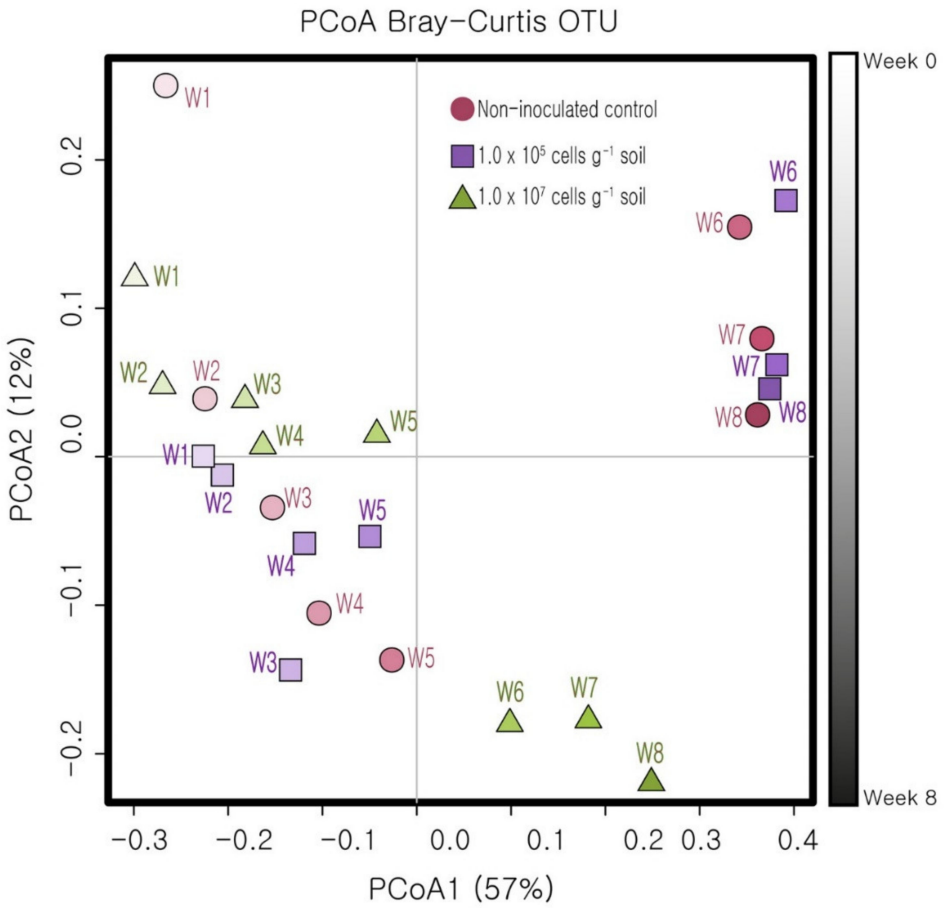

Figure 5. Principal coordinate analysis of 16S rRNA genes of total bacteria based on the Bray–Curtis similarity index at 97% identity (operational taxonomic unit level) for eight weeks (W1–W8). PCoA1 and PCoA2 explained 57% and 12% of the variance, respectively.

We employed the PICRUSt program to predict the function of the soil bacterial community based on 16S rRNA gene data (Figure 6). The PICRUSt functional analysis showed that pathways related to germination and sporulation were overrepresented before six weeks in samples that received an application of KNU-07 (1.0×10^7 cells g^{-1} soil) (Figure 6). After six weeks, the pathways that were positively impacted by the application of KNU-07 (1.0×10^7 cells g^{-1} soil) were energy metabolism and metabolism of cofactors and vitamins (Figure 6).

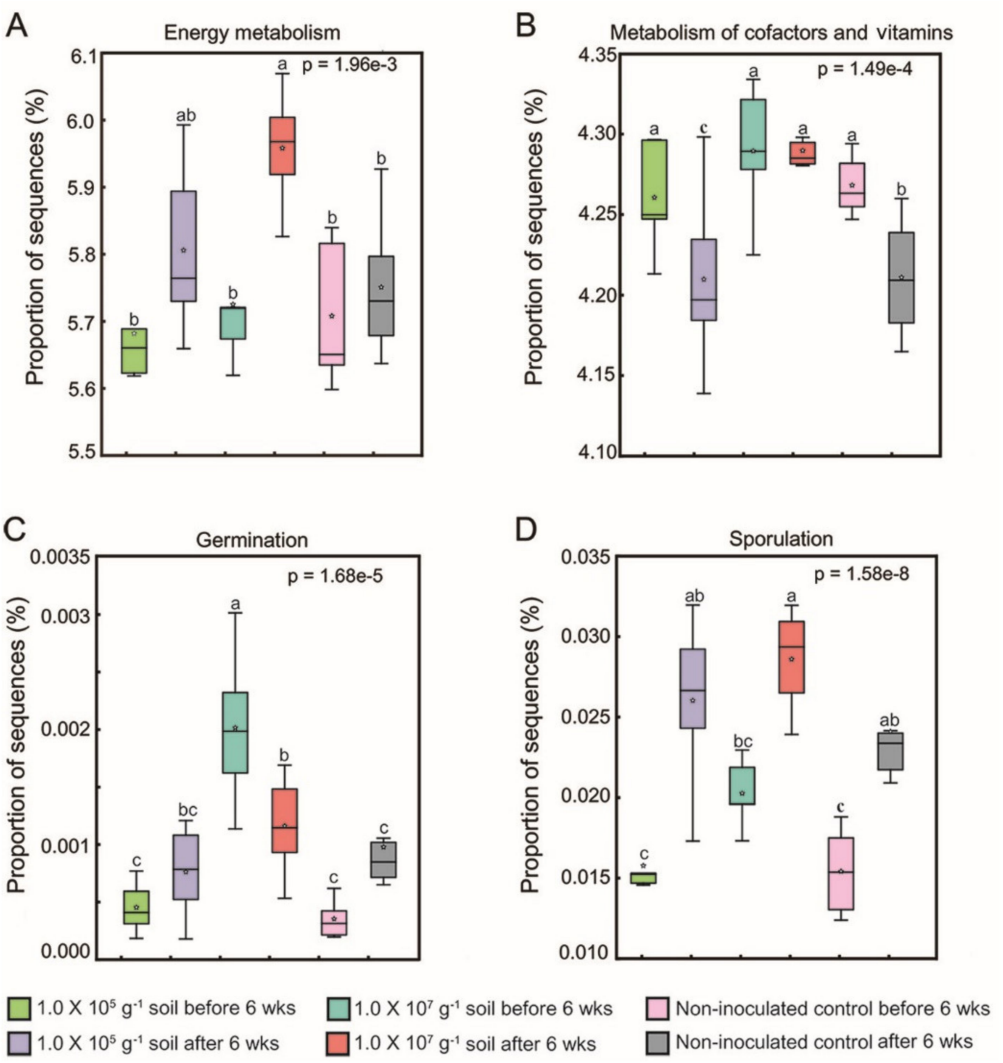

Figure 6. Predicted metabolic function from 16S rRNA gene sequences of soil bacterial community collected from KNU-07 bacterized and non-bacterized samples using PICRUSt and STAMP analysis before and after six weeks inoculation with the indicated concentrations of *B. thuringiensis* KNU-07. (**A**) Energy metabolism, (**B**) metabolism of cofactors and vitamins, (**C**) germination, and (**D**) sporulation. Non-inoculated control (Control). Non-inoculated plants served as controls. Mean values having different letters in each parameter are significantly different ($p \leq 0.05$).

3.4. Tracking of KNU-07 Population Using qPCR

The results of qPCR data showed soil treated with KNU-07 at higher concentrations had the highest abundance of KNU-07 throughout the experiments. As expected, KNU-07 cells were not detected in any non-inoculated control soil at any time (Figure 7). The abundance of KNU-07 decreased over time, regardless of the initial concentration of the KNU-07 inoculum (Figure 7). KNU-07 cells were detected long after inoculation (six weeks) from soils initially inoculated with a high concentration of KNU-07 (1.0×10^7 cells g^{-1} soil). However, KNU-07 cells were detected in soil initially inoculated with a low concentration of KNU-07 (1.0×10^5 cells g^{-1} soil) only within 3 weeks of inoculation (Figure 7). After eight weeks of inoculation, KNU-07 cells were not detected in any sample. We also investigated the total bacteria population using the 16S rRNA gene to determine whether there was a decrease in the total bacteria population, as was observed for KNU-07. The results of qPCR data showed that the abundance of total bacteria in all samples, including controls, increased slightly over time (Figure 7).

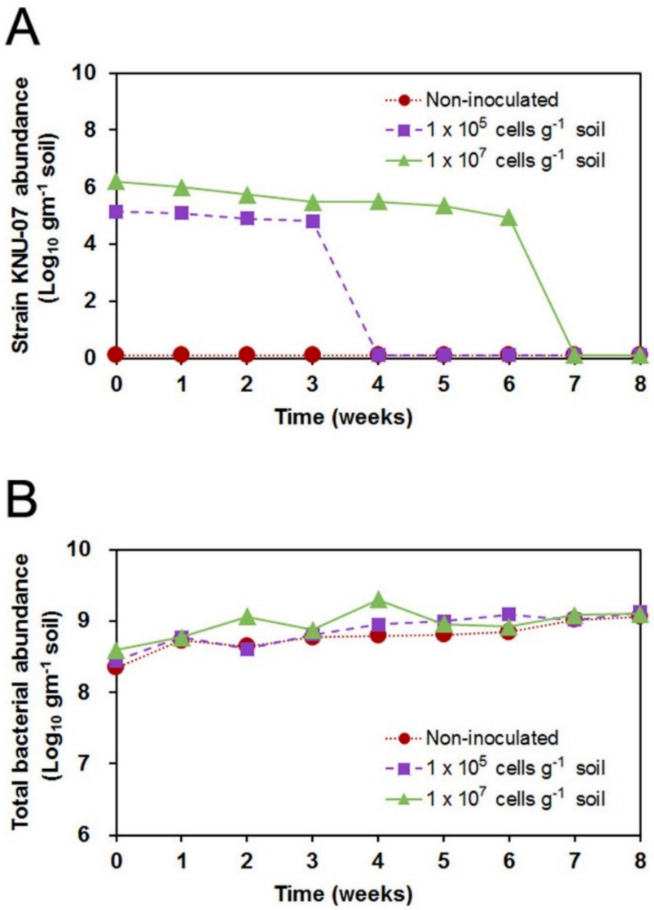

Figure 7. Abundance of (**A**) *B. thuringiensis* KNU-07 based on unique sequence copies and (**B**) total bacteria based on 16S rRNA gene copies in the soil over eight weeks post-inoculation using qPCR.

4. Discussion

In this study, we assessed the effect of KNU-07 on the growth of pepper plants and the soil bacterial community and designed a strain-specific primer pair to track the population dynamics of KNU-07 in soil using a qPCR-based method. Similar to the results of our in vitro assays, IAA production, siderophore production, phosphate solubilization, and urease activity by several strains of *B. thuringiensis* have been previously reported [12–14]. An increase in IAA production in the presence of L-tryptophan may attributed to the nature of the strain to utilize L-tryptophan as a physiological precursor [35]. Our in vivo assays also showed that KNU-07 promoted the growth of pepper plants after inoculation into the soil. Strains of *B. thuringiensis* have been used to promote the growth of plants, and our findings are consistent with these reports [36–38]. Previous studies reported that PGPR strains possessing siderophore production play a great role in helping plants to acquire iron for plant growth [13]. In addition, IAA production, phosphate solubilization, and urease activities play important roles in enhancing nutrient and water uptake and thereby enhance plant growth [12,14].

The change in soil bacterial community structure due to the presence of KNU-07 was less visible before six weeks post-inoculation. However, the community structure was separated after six weeks. Ke et al. [39] reported that the inoculation of soil with *Pseudomonas stutzeri* A1501 significantly changed the indigenous soil bacterial community structure after 2 months of inoculation, and our findings are consistent with this report. Similarly, Wang et al. [40] discussed the significant effect of bio-inoculants on soil microbial communities. In this study, the abundance of the Ellin 329 and Rhizobiales orders were higher in all samples. This may be due to the loss of Acidobacteriales [41,42]. There was also a change in soil microbial community structure over time. In our study, KNU-07-bacterized plants

exhibited superior growth relative to control plants. Plant age has been reported to influence the dynamics of the soil microbiome [43,44], and our findings are consistent with these reports.

Predicting the function of the total bacterial community provides information about its interaction with the surrounding environment [45]. Hence, we employed PICRUSt to predict changes in the function of the soil microbiota due to KNU-07 inoculation. Several metabolic pathways that facilitate growth in plants were overrepresented following application KNU-07 (1.0×10^7 cells g^{-1} soil). He et al. [46] reported that rhizobacteria inoculation had beneficial effects on the function of the bacterial community. Predicted metabolic functions related to sporulation and germination were significantly affected during the first week after inoculation with KNU-07 at the highest concentration. The elevated abundance of predicted genes related to sporulation and germination might arise from the inoculated KNU-07, which belongs to the Bacillales order. Sporulation is a survival mechanism of *Bacillus* spp. in response to unfavorable environmental conditions [47]. More importantly, after six weeks post-inoculation, KNU-07 pathways related to energy metabolism and the metabolism of cofactors and vitamins were found to be overrepresented. This might give the pepper plants growing in inoculated soil better nutrition and plant growth [39,41,48].

Quantifying the abundance of a microbial inoculant in the soil is one of the best strategies for tracking [20,24]. Tracking helps to investigate the potential of inoculated microbes because PGPR is based on their persistence in the soil. Tracking bio-inoculants in the soil has been performed using different methods, including dilution plating and microscopy [49,50]. However, such methods can be laborious, time-consuming, and limited to sterile conditions [51]. Interestingly, a few recent studies proposed the possibility of tracking bacterial populations in field samples by using strain-specific primers in qPCR-based protocols [24,52]. To the best of our knowledge, this study is the first report of monitoring *B. thuringiensis* abundance in non-sterile soil using a single strain-specific primer pair in a qPCR-based method. The abundance of KNU-07 was relatively stable during the first two weeks post-inoculation and decreased over time regardless of the initial KNU-07 concentration. Coy et al. [53] reported that the population of *Bacillus sphaericus* drastically declined after six weeks post-inoculation. The bacterial population of antagonistic bacteria has also been reported to decline over time [54]. These decreases in the abundance of soil bio-inoculants might be attributed to physical and biological factors found in the soil environment [55]. Another factor that might cause a decrease in the abundance of KNU-07 may be microbial competition [53,56]. In this study, the amplicon sequence data of 16S rRNAs revealed that there was a gradual increase in the abundance of the total bacterial over time.

The design of a strain-specific primer pair and being able to track the strain in the soil by qPCR offers important information about the fate of PGPR under non-sterile soil conditions, which is an important step in registering a microbe as a PGPR product. Nevertheless, further studies are needed to identify ways to increase the survival of KNU-07 under different soil environmental conditions.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/4/551/s1, Figure S1: Effectiveness of the single primer pair for specific detection of *B. thuringiensis* KNU-07. (A) Lane M: Doctor protein 1 kb plus ladder, lane 1: negative control, lane 2: KNU-07, lane 3–30: different bacterial strains samples (Table S1). (B) Lane M: Doctor protein 1 kb plus ladder, lane 1: negative control, lane 2: KNU-07, lane 3–30: soil samples isolated from different locations (Table S2). Primer pair KNU07F/ KNU07R without template KNU-07 DNA served as the negative control, Figure S2. Potential of some *Bacillus* spp. (1–8) and KNU-07 (9) for indole acetic acid production (A), siderophore activity (B), urease activity (C) and phosphatase activity (D). *1* = *Bacillus licheniformis* KACC 10476, *2* = *Bacillus megaterium* KACC 10482, *3* = *Bacillus polymyxa* KACC 10485, *4* = *Bacillus subtilis* KACC 10854, *5* = *Bacillus pumilus* KACC 10917, *6* = *Bacillus macerans* KACC 11233, *7* = *Bacillus amyloliquefaciens* KACC 12067, *8* = *Bacillus velezensis* KACC 14004. Table S1: Bacterial strains used in this study, Table S2: Sources of soil samples used for in vitro PCR assays, Table S3. Statistical analysis of bacterial community structure at an operational taxonomic unit level in the last three weeks.

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References

1. Compant, S.; Clément, C.; Sessitsch, A. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* **2010**, *42*, 669–678. [CrossRef]
2. Anusha, B.G.; Gopalakrishnan, S.; Naik, M.K.; Sharma, M. Evaluation of *Streptomyces* spp. and *Bacillus* spp. for biocontrol of *Fusarium* wilt in chickpea (*Cicer arietinum* L.). *Arch. Phytopathol. Plant Prot.* **2019**, *52*, 417–442. [CrossRef]
3. Gamez, R.; Cardinale, M.; Montes, M.; Ramirez, S.; Schnell, S.; Rodriguez, F. Screening, plant growth promotion and root colonization pattern of two rhizobacteria (*Pseudomonas fluorescens* Ps006 and *Bacillus amyloliquefaciens* Bs006) on banana cv. Williams (*Musa acuminata* Colla). *Microbiol. Res.* **2019**, *220*, 12–20. [CrossRef] [PubMed]
4. Mhatre, P.H.; Karthik, C.; Kadirvelu, K.; Divya, K.L.; Venkatasalam, E.P.; Srinivasan, S.; Ramkumar, G.; Saranya, C.; Shanmuganathan, R. Plant growth promoting rhizobacteria (PGPR): A potential alternative tool for nematodes bio-control. *Biocatal. Agric. Biotechnol.* **2019**, *17*, 119–128. [CrossRef]
5. Chowdhury, S.P.; Hartmann, A.; Gao, X.W.; Borriss, R. Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42—A review. *Front. Microbiol.* **2015**, *6*, 780. [CrossRef]
6. Shafi, J.; Tian, H.; Ji, M. *Bacillus* species as versatile weapons for plant pathogens: A review. *Biotechnol. Biotechnol. Equip.* **2017**, *31*, 446–459. [CrossRef]
7. Babu, A.G.; Kim, J.D.; Oh, B.T. Enhancement of heavy metal phytoremediation by *Alnus firma* with endophytic *Bacillus thuringiensis* GDB-1. *J. Hazard. Mater.* **2013**, *250*, 477–483. [CrossRef]
8. Azizoglu, U. *Bacillus thuringiensis* as a Biofertilizer and Biostimulator: A Mini-Review of the Little-Known Plant Growth-Promoting Properties of Bt. *Curr. Microbiol.* **2019**, *76*, 1379–1385. [CrossRef]
9. Singh, S.; Kumar, V.; Sidhu, G.K.; Datta, S.; Dhanjal, D.S.; Koul, B.; Janeja, H.S.; Singh, J. Plant growth promoting rhizobacteria from heavy metal contaminated soil promote growth attributes of *Pisum sativum* L. *Biocatal. Agric. Biotechnol.* **2019**, *17*, 665–671. [CrossRef]
10. Wang, M.; Chen, S.; Han, Y.; Chen, L.; Wang, D. Responses of soil aggregates and bacterial communities to soil-Pb immobilization induced by biofertilizer. *Chemosphere* **2019**, *220*, 828–836. [CrossRef]
11. You, C.; Zhang, C.; Kong, F.; Feng, C.; Wang, J. Comparison of the effects of biocontrol agent *Bacillus subtilis* and fungicide metalaxyl-mancozeb on bacterial communities in tobacco rhizospheric soil. *Ecol. Eng.* **2016**, *91*, 119–125. [CrossRef]
12. Wang, T.; Liu, M.Q.; Li, H.X. Inoculation of phosphate-solubilizing bacteria *Bacillus thuringiensis* B1 increases available phosphorus and growth of peanut in acidic soil. *Acta Agric. Scand. Sect. B Soil Plant Sci.* **2014**, *64*, 252–259.
13. Hollensteiner, J.; Wemheuer, F.; Harting, R.; Kolarzyk, A.M.; Diaz Valerio, S.M.; Poehlein, A.; Brzuszkiewicz, E.B.; Nesemann, K.; Braus-Stromeyer, S.A.; Braus, G.H.; et al. *Bacillus thuringiensis* and *Bacillus weihenstephanensis* inhibit the growth of phytopathogenic *Verticillium* species. *Front. Microbiol.* **2017**, *7*, 2171. [CrossRef] [PubMed]
14. Khan, N.; Bano, A.; Rahman, M.A.; Guo, J.; Kang, Z.; Babar, M.A. Comparative physiological and metabolic analysis reveals a complex mechanism involved in drought tolerance in chickpea (Cicer arietinum L.) induced by PGPR and PGRs. *Sci. Rep.* **2019**, *9*, 2097. [CrossRef]
15. Gange, A.C.; Gadhave, K.R. Plant growth-promoting rhizobacteria promote plant size inequality. *Sci. Rep.* **2018**, *8*, 13828. [CrossRef]

16. Goswami, D.; Thakker, J.N.; Dhandhukia, P.C. Portraying mechanics of plant growth promoting rhizobacteria (PGPR): A review. *Cogent Food Agric.* **2016**, *2*, 1127500. [CrossRef]
17. Cao, Y.; Pi, H.; Chandrangsu, P.; Li, Y.; Wang, Y.; Zhou, H.; Xiong, H.; Helmann, J.D.; Cai, Y. Antagonism of Two Plant-Growth Promoting *Bacillus velezensis* Isolates Against *Ralstonia solanacearum* and *Fusarium oxysporum*. *Sci. Rep.* **2018**, *8*, 4360. [CrossRef]
18. Mosimann, C.; Oberhänsli, T.; Ziegler, D.; Nassal, D.; Kandeler, E.; Boller, T.; Mäder, P.; Thonar, C. Tracing of two *Pseudomonas* strains in the root and rhizoplane of maize, as related to their plant growth-promoting effect in contrasting soils. *Front. Microbiol.* **2017**, *7*, 2150. [CrossRef]
19. Aufrecht, J.A.; Timm, C.M.; Bible, A.; Morrell-Falvey, J.L.; Pelletier, D.A.; Doktycz, M.J.; Retterer, S.T. Microfluidics: Quantifying the Spatiotemporal Dynamics of Plant Root Colonization by Beneficial Bacteria in a Microfluidic Habitat (Adv. Biosys. 6/2018). *Adv. Biosyst.* **2018**, *2*, 1800048. [CrossRef]
20. Rilling, J.I.; Acuña, J.J.; Nannipieri, P.; Cassan, F.; Maruyama, F.; Jorquera, M.A. Current opinion and perspectives on the methods for tracking and monitoring plant growth-promoting bacteria. *Soil Biol. Biochem.* **2019**, *130*, 205–219. [CrossRef]
21. Goulart, M.C.; Cueva-Yesquén, L.G.; Hidalgo Martinez, K.J.; Attili-Angelis, D.; Fantinatti-Garboggini, F. Comparison of specific endophytic bacterial communities in different developmental stages of *Passiflora incarnata* using culture-dependent and culture-independent analysis. *Microbiologyopen* **2019**, *8*, e896. [CrossRef] [PubMed]
22. Kraková, L.; Šoltys, K.; Otlewska, A.; Pietrzak, K.; Purkrtová, S.; Savická, D.; Puškárová, A.; Bučková, M.; Szemes, T.; Budiš, J.; et al. Comparison of methods for identification of microbial communities in book collections: Culture-dependent (sequencing and MALDI-TOF MS) and culture-independent (Illumina MiSeq). *Int. Biodeterior. Biodegrad.* **2018**, *131*, 51–59. [CrossRef]
23. Stets, M.I.; Alqueres, S.; Souza, E.M.; Pedrosa, F.O.; Schmid, M.; Hartmann, A.; Cruz, L.M. Quantification of *Azospirillum brasilense* FP2 bacteria in wheat roots by strain-specific quantitative PCR. *Appl. Environ. Microbiol.* **2015**, *81*, 6700–6709. [CrossRef]
24. Mendis, H.C.; Thomas, V.P.; Schwientek, P.; Salamzade, R.; Chien, J.T.; Waidyarathne, P.; Kloepper, J.; De La Fuente, L. Strain-specific quantification of root colonization by plant growth promoting rhizobacteria *Bacillus firmus* I-1582 and *Bacillus amyloliquefaciens* QST713 in non-sterile soil and field conditions. *PLoS ONE* **2018**, *13*, e0193119. [CrossRef]
25. Ficetola, G.F.; Coissac, E.; Zundel, S.; Riaz, T.; Shehzad, W.; Bessière, J.; Taberlet, P.; Pompanon, F. An in silico approach for the evaluation of DNA barcodes. *BMC Genom.* **2010**, *11*, 434. [CrossRef] [PubMed]
26. Gordon, S.A.; Weber, R.P. Colorimetric estimation of indoleacetic acid. *Plant Physiol.* **1951**, *26*, 192–195. [CrossRef]
27. Kurtzman, C.P.; Fell, J.W.; Boekhout, T.; Robert, V. Methods for isolation, phenotypic characterization and maintenance of yeasts. In *The Yeasts*; Elsevier: London, UK; Amsterdam, The Netherlands, 2011; ISBN 9780444521491.
28. Pérez-Miranda, S.; Cabirol, N.; George-Téllez, R.; Zamudio-Rivera, L.S.; Fernández, F.J. O-CAS, a fast and universal method for siderophore detection. *J. Microbiol. Methods* **2007**, *31*, 446–459. [CrossRef]
29. Kyaw, A.; Maung-U, K.; Toe, T. Determination of inorganic phosphate with molybdate and Triton X-100 without reduction. *Anal. Biochem.* **1985**, *145*, 230–234. [CrossRef]
30. Anderson, M.J. A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* **2001**, *26*, 32–46.
31. CLARKE, K.R. Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol.* **1993**, *18*, 117–143. [CrossRef]
32. Langille, M.G.I.; Zaneveld, J.; Caporaso, J.G.; McDonald, D.; Knights, D.; Reyes, J.A.; Clemente, J.C.; Burkepile, D.E.; Vega Thurber, R.L.; Knight, R.; et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* **2013**, *31*, 814–821. [CrossRef] [PubMed]
33. Parks, D.H.; Tyson, G.W.; Hugenholtz, P.; Beiko, R.G. STAMP: Statistical analysis of taxonomic and functional profiles. *Bioinformatics* **2014**, *30*, 3123–3124. [CrossRef] [PubMed]
34. SAS Institute. *SAS Version 9.4*; SAS Institute Inc.: Cary, NC, USA, 2012.

35. Spaepen, S.; Vanderleyden, J.; Remans, R. Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol. Rev.* **2007**, *31*, 425–448. [CrossRef] [PubMed]
36. Tagele, S.B.; Lee, H.G.; Kim, S.W.; Lee, Y.S. Phenazine and 1-undecene producing *Pseudomonas chlororaphis* subsp. *aurantiaca strain KNU17Pc1 for growth promotion and disease suppression in Korean maize cultivars. J. Microbiol. Biotechnol.* **2019**, *29*, 66–78.
37. Viljoen, J.J.F.; Labuschagne, N.; Fourie, H.; Sikora, R.A. Biological control of the root-knot nematode *Meloidogyne incognita* on tomatoes and carrots by plant growth-promoting rhizobacteria. *Trop. Plant Pathol.* **2019**, *44*, 284–291. [CrossRef]
38. Vidal-Quist, J.C.; Rogers, H.W.J.; Mahenthiralingam, E.; Berry, C. *Bacillus thuringiensis* colonises plant roots in a phylogeny-dependent manner. *FEMS Microbiol. Ecol.* **2013**, *86*, 474–489. [CrossRef]
39. Ke, X.; Feng, S.; Wang, J.; Lu, W.; Zhang, W.; Chen, M.; Lin, M. Effect of inoculation with nitrogen-fixing bacterium *Pseudomonas stutzeri* A1501 on maize plant growth and the microbiome indigenous to the rhizosphere. *Syst. Appl. Microbiol.* **2019**, *42*, 248–260. [CrossRef]
40. Wang, J.; Li, Q.; Xu, S.; Zhao, W.; Lei, Y.; Song, C.; Huang, Z. Traits-based integration of multi-species inoculants facilitates shifts of indigenous soil bacterial community. *Front. Microbiol.* **2018**, *9*, 1692. [CrossRef]
41. Bouskill, N.J.; Lim, H.C.; Borglin, S.; Salve, R.; Wood, T.E.; Silver, W.L.; Brodie, E.L. Pre-exposure to drought increases the resistance of tropical forest soil bacterial communities to extended drought. *ISME J.* **2013**, *7*, 384–394. [CrossRef]
42. Harbison, A.B.; Carson, M.A.; Lamit, L.J.; Basiliko, N.; Bräuer, S.L. A novel isolate and widespread abundance of the candidate alphaproteobacterial order (Ellin 329), in southern Appalachian peatlands. *FEMS Microbiol. Lett.* **2016**, *363*, fnw151. [CrossRef]
43. Van Overbeek, L.; Van Elsas, J.D. Effects of plant genotype and growth stage on the structure of bacterial communities associated with potato (*Solanum tuberosum* L.). *FEMS Microbiol. Ecol.* **2008**, *64*, 283–296. [CrossRef] [PubMed]
44. Degrune, F.; Theodorakopoulos, N.; Colinet, G.; Hiel, M.P.; Bodson, B.; Taminiau, B.; Daube, G.; Vandenbol, M.; Hartmann, M. Temporal dynamics of soil microbial communities below the seedbed under two contrasting tillage regimes. *Front. Microbiol.* **2017**, *8*, 1127. [CrossRef] [PubMed]
45. Han, L.; Wang, Z.; Li, N.; Wang, Y.; Feng, J.; Zhang, X. *Bacillus amyloliquefaciens* B1408 suppresses Fusarium wilt in cucumber by regulating the rhizosphere microbial community. *Appl. Soil Ecol.* **2019**, *136*, 55–66. [CrossRef]
46. He, Y.; Pantigoso, H.A.; Wu, Z.; Vivanco, J.M. Co-inoculation of *Bacillus* sp. and *Pseudomonas putida* at different development stages acts as a biostimulant to promote growth, yield and nutrient uptake of tomato. *J. Appl. Microbiol.* **2019**, *127*, 196–207. [CrossRef]
47. Omony, J.; de Jong, A.; Krawczyk, A.O.; Eijlander, R.T.; Kuipers, O.P. Dynamic sporulation gene co-expression networks for *Bacillus subtilis* 168 and the food-borne isolate *Bacillus amyloliquefaciens*: A transcriptomic model. *Microb. Genom.* **2018**, *4*, e000157. [CrossRef] [PubMed]
48. Mendes, L.W.; Kuramae, E.E.; Navarrete, A.A.; Van Veen, J.A.; Tsai, S.M. Taxonomical and functional microbial community selection in soybean rhizosphere. *ISME J.* **2014**, *8*, 1577–1587. [CrossRef]
49. Taute, K.M.; Gude, S.; Tans, S.J.; Shimizu, T.S. High-throughput 3D tracking of bacteria on a standard phase contrast microscope. *Nat. Commun.* **2015**, *6*, 1–9. [CrossRef]
50. Banik, A.; Mukhopadhaya, S.K.; Sahana, A.; Das, D.; Dangar, T.K. Fluorescence resonance energy transfer (FRET)-based technique for tracking of endophytic bacteria in rice roots. *Biol. Fertil. Soils* **2016**, *52*, 277–282. [CrossRef]
51. Segarra, G.; Puopolo, G.; Porcel-Rodríguez, E.; Giovannini, O.; Pertot, I. Monitoring *Lysobacter capsici* az78 using strain specific qpcr reveals the importance of the formulation for its survival in vineyards. *FEMS Microbiol. Lett.* **2016**, *363*, fnv243. [CrossRef]
52. Ricketts, C.; Pickler, L.; Maurer, J.; Ayyampalayam, S.; García, M.; Ferguson-Noel, N.M. Identification of strain-specific sequences that distinguish a *Mycoplasma gallisepticum* vaccine strain from field isolates. *J. Clin. Microbiol.* **2017**, *55*, 244–252. [CrossRef]
53. Coy, R.M.; Held, D.W.; Kloepper, J.W. Rhizobacterial colonization of bermudagrass by *Bacillus* spp. in a Marvyn loamy sand soil. *Appl. Soil Ecol.* **2019**, *141*, 10–17. [CrossRef]

54. Khabbaz, S.E.; Abbasi, P.A. Isolation, characterization, and formulation of antagonistic bacteria for the management of seedlings damping-off and root rot disease of cucumber. *Can. J. Microbiol.* **2014**, *60*, 25–33. [CrossRef] [PubMed]
55. Andreote, F.D.; De Araújo, W.L.; De Azevedo, J.L.; Van Elsas, J.D.; Da Rocha, U.N.; Van Overbeek, L.S. Endophytic colonization of potato (*Solanum tuberosum* L.) by a novel competent bacterial endophyte, *Pseudomonas putida* strain P9, and its effect on associated bacterial communities. *Appl. Environ. Microbiol.* **2009**, *75*, 3396–3406. [CrossRef] [PubMed]
56. Ishizawa, H.; Kuroda, M.; Inoue, K.; Inoue, D.; Morikawa, M.; Ike, M. Colonization and competition dynamics of plant growth-promoting/inhibiting Bacteria in the phytosphere of the Duckweed Lemna minor. *Microb. Ecol.* **2019**, *77*, 440–450. [CrossRef] [PubMed]