

## Article

# Isolation and Characterization of Plant Growth-Promoting Compost Bacteria That Improved Physiological Characteristics in Tomato and Lettuce Seedlings

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**Abstract:** Currently, agricultural systems are inadequate to meet the demand of the population, coupled with the constant degradation of natural resources. Therefore, it is necessary to explore alternatives to increase the productivity and quality of crops with minimal environmental impact. The use of plant growth-promoting bacteria can provide solutions to some agri-environmental problems and replace or minimize conventional agricultural practices. In this study, a *Bacillus pumilus* strain with plant growth-promoting properties was isolated from mature compost. In vitro, the ability of *Bacillus pumilus* to solubilize phosphate, inhibit the growth of phytopathogenic fungi, and its effect on the germination of tomato and lettuce seeds was evaluated. In vivo, its effect on stem thickness, height, and the number of leaves of tomato and lettuce seedlings was studied. The results show that, in vitro, *Bacillus pumilus* solubilizes phosphate, inhibits the growth of the fungus *Fusarium oxysporum*, and increases the germination percentage of tomato seeds. The results, in vivo, demonstrate that the bacteria increases the stem thickness of tomato seedlings, while, in lettuce, it increases the stem thickness and the number of leaves. The outcome implies that *Bacillus pumilus* has properties as a plant growth promoter and can be used as a promising inoculant to enhance the growth of tomato and lettuce seedlings.

**Keywords:** antagonism; cow manure compost; phosphate solubilizing bacteria; plant growth-promoting bacteria

## 1. Introduction

Today, agriculture faces the challenge of producing more food in a smaller area in order to meet the growing demand of the population and the impact of constant soil degradation, climate change, and water scarcity, which decrease crop yield [1]. Therefore, it is important to implement different strategies for the rational use of natural resources. One of these strategies is the application of plant growth-promoting bacteria (PGPB) in crops. These bacteria have phytostimulant connections; they also intervene in biochemical cycles, actively participate in the availability of nutrients, and have properties as biocontrol agents [2,3]. In addition, some bacteria solubilize phosphate rock and other sources of inorganic phosphorus in the soil; for this reason, they are called phosphate-solubilizing bacteria. This activity is relevant because a large amount of the P contributed by phosphate fertilizers loses efficiency due to different biochemical processes such as sorption, fixation, and immobilization, which in turn causes high rates of P application in crops. The inefficient use of phosphate fertilizers can modify the state of the nutrients in the soil, the availability for the absorption of the crops, and the microbial communities [4]. Finally, phosphate solubilizing bacteria can be an alternative for increasing the phosphorus available to plants [5,6].

Compost use is constantly increasing since it is considered a potential alternative for controlling soil pathogens and increasing plant growth, which is safer for overall health and the environment. Several studies have shown that the properties of compost are governed by mechanisms of microbial origin. That is, plant growth and phytopathogens control occur due to the presence of some PGPB in the bio-oxidative composting process of organic waste to produce stable material rich in humic substances [7–9].

The inoculation of PGPB in agricultural lands contributes to improvement in the microbiota and the physical properties of the soil, which in turn decreases production cost and increases the yield of crops [10]. This technology promises to provide solutions to some agri-environmental problems since it promotes plant growth and increases the availability and absorption of nutrients through the production of phytohormones, antagonism against phytopathogens, or the activation of systemic resistance [11].

Diverse studies show that PGPB associated with the rhizosphere of plants have beneficial effects on plant growth, such as *Bacillus* [12,13]. Several species have been detected inside this genus that show potential as promoters of plant growth [14,15]. *Bacillus pumilus* promotes growth in beans [16] and in different varieties of rice [17], mustard, and radish [18]. Additionally, it improves the quality of tomato fruit [19], mainly due to its potential to increase the absorption of nutrients by promoting root growth. The inoculation of *B. pumilus* in lettuce plants considerably increases vigor, average head weight, and height compared with other PGPB [20]. However, the positive interaction of *B. pumilus* with crops depends on the plant genotype, soil nutrition, inoculant density, and environmental conditions, which significantly affects the capacity of PGPB to improve plant growth under field conditions. [21]. Moreover, *B. pumilus* transforms the organic P available to plants [22].

Several studies have focused on evaluating the activities of PGPB with commercial interest. However, the evaluation of a PGPB isolated from compost with phosphorus solubilizing activity, an antagonist of pathogens and promotion of plant growth, has had very little evidence regarding its properties.

The main objective of this study was to isolate and characterize plant growth-promoting bacteria from mature compost to evaluate, *in vitro*, the bacteria's ability to solubilize phosphate and inhibit the growth of phytopathogenic fungi and subsequently to also determine the effect of the isolated bacteria on germination, height, stem thickness, and the number of leaves of lettuce and tomato seedlings.

## 2. Materials and Methods

### 2.1. Isolation and Identification of PGPB from Mature Compost

#### 2.1.1. Isolation of Bacteria

The samples were obtained from a 75-day-old compost. The compost was prepared from bovine manure from a barn located at the Amazcala campus of the Engineering Faculty of the Autonomous University of Querétaro, municipality of El Marqués, Querétaro, México.

The compost sampling was carried out by separating the compost at depths of 0–25 cm, 50–75 cm, and 100–125 cm to the left, center, and right. Samples were placed in sterile sealed plastic bags and homogenized, to later perform the bacterial isolation.

For isolation purposes, 10 g of each sample was dissolved in 90 mL of sterile distilled water and 0.1% Tween 20 and incubated with constant shaking. Each solution was serially diluted to  $10^6$ ; a 1 mL aliquot was taken and inoculated onto Pikovskaya agar plates to select a phosphate-solubilizing bacteria. The plates were incubated at  $28 \pm 2$  °C for a period of 72 h. When a translucent halo was visualized on Pikovskaya agar, the bacterial strain with the higher halo was selected, inoculated into Luria Bertani (LB) broth, and Gram-stained [23].

Then, the selected colonies were streaked three times on fresh nutrient agar plates to obtain a pure isolate, and the purified bacterial isolates were stored at 4 °C in 20% glycerol. Subsequently, some pure isolates were lyophilized for long-term storage.

### 2.1.2. Molecular Characterization

Bacterial genomic DNA was extracted using Kirby's technique, which consists of lysing cells and solubilizing DNA [24]. After DNA extraction, it was amplified with the 16S rRNA gene. Sequencing was carried out at the National Laboratory of Agricultural, Medical, and Environmental Biotechnology of the IPICYT using the dideoxynucleotide method marked with a Genetic Analyzer 3130 sequencer. The DNA sequencing of the 16S ribosomal gene obtained from the amplicon was compared with others in the GenBank database using the NCBI BLAST nucleotide sequence program on the website <https://blast.ncbi.nlm.nih.gov/Blast.cgi> (accessed on 25 November 2021).

The complete sequence was deposited in NCBI GenBank with access number SUB5832921 Seq2 MN067217.

### 2.1.3. Phosphate Solubilization

An inoculum of *Bacillus pumilus* grown in LB broth was taken, placed in Pikovskaya medium, and incubated at  $28 \pm 2$  °C for 5 days [25]. On the plates, the formation of clear areas around the colonies was observed. In order to determine the phosphate solubilizing activity of the bacteria, the phosphate solubilization index (PSI) was calculated with Equation (1) [26], and the relative solubilization efficiency (RSE) was calculated with Equation (2) [27]. The study was triplicated for comparison purposes.

$$\text{PSI} = \frac{\text{Halo zone diameter}}{\text{Colony diameter}} \quad (1)$$

$$\text{RSE} = \frac{\text{Solubilization mean diameter}}{\text{Colony mean diameter}} \times 100 \quad (2)$$

### 2.1.4. Antifungal Activity against *Fusarium oxysporum* and *Sclerotium cepivorum*

An inoculum of *B. pumilus* was prepared and standardized in LB broth at a concentration of  $1 \times 10^9$  CFU/mL. On the other hand, a disk of *F. oxysporum* or *S. cepivorum* was placed in the center of potato dextrose agar (PDA) plates, and a line was made with 25 µL of the inoculum 15 mm from each side of the disk. As a control, a fungus strain grown on PDA was used without any treatment. Treatments were incubated for 12 days at room temperature [28]. The study was carried out in triplicate.

After 12 days, the mycelial growth of the pathogenic fungi was measured, and the inhibition index was calculated with Equation (3).

$$I = \frac{\text{Control mycelium diameter}}{\text{Treatment mycelium diameter}} \times 100 \quad (3)$$

*I* is the inhibition percentage.

## 2.2. Evaluating the Effect of *Bacillus Pumilus* on Plant Growth

### 2.2.1. Preparation of Inoculum and Seed Selection

*Bacillus pumilus* was inoculated in 100 mL of LB broth and incubated at  $28 \pm 2$  °C with constant rotational shaking of 150 rpm for 48 h until the exponential growth phase [29]. Inoculum standardization was carried out through dilutions with 0.9% sterile saline solution until obtaining a bacterial cell density of  $1 \times 10^9$  CFU/mL.

To check the bacterial density, ten serial dilutions of the inoculum were made and 1 mL of each dilution was taken and inoculated in nutrient agar plates. The plates were incubated at  $28 \pm 2$  °C for 24 h. Finally, cell growth was counted and recorded [30].

For the germination test, commercial seeds of saladette-type tomato (*Solanum lycopersicum* L.) and lettuce (*Lactuca sativa* L. var. *Longifolia*) were used. The seeds were disinfected

with 70% ethanol (volume/volume) for one minute, then 2.5% sodium hypochlorite for 20 min, and washed three times with sterile distilled water. Floating seeds were discarded.

$$\text{germination \%} = \frac{\text{Germinated seeds}}{\text{Total seeds}} \times 100 \quad (4)$$

### 2.2.2. Effect of *Bacillus pumilus* on the Germination of Lettuce and Tomato Seeds

Squared and sterilized filter paper was placed inside Petri dishes. A tomato or lettuce seed was placed in each space until there were 45 seeds per Petri dish. The seeds were moistened with 5 mL of sterile distilled water and 0.1 mL of *B. pumilus* per seed. The plates were sealed and kept at room temperature, approximately  $20 \pm 5$  °C, to maintain for 20 days the temperature conditions of the region where the experiments were performed. Control was 45 uninoculated seeds in the Petri dish. The amount of germinated seeds was recorded daily, and after 20 days, the germination percentage was calculated with Equation (4) [23]. Finally, the test had a completely randomized one-factor design with three replications.

### 2.2.3. Effect of *Bacillus pumilus* on Tomato and Lettuce Seedlings

Tomato and lettuce seeds previously disinfected were sown in seedbeds with sterile peat. Under aseptic conditions, one seed was placed per cavity. Daily irrigation with sterile distilled water was maintained until the seedlings emerged.

Inoculation with *Bacillus pumilus* started seven days after sowing, and 1 mL of standardized bacterial inoculum was added to each cavity, close to the seedling roots every seven days for five weeks [31]. Non-inoculated seedlings were used as a control.

The experiment was established in a greenhouse at an average temperature of 21.5 °C, a humidity of 81.9%, and a photoperiod of 10 h, approximately. The seedlings were watered every three days with sterile distilled water at field capacity. At the end of the experiment (5 weeks after sowing), the height and thickness of the stem were measured, as well as the chlorophyll content and the number of leaves. For the measurements, a digital caliper was used—range 0–6"/0–150 mm, resolution 0.0005"/0.01 mm, Surtek brand. Additionally, chlorophyll was measured with a SPAD-502 Plus Chlorophyll Meter. Finally, the experiment had a completely randomized univariate design with three replications, with experimental units of 10 seedlings.

## 2.3. Statistical Analysis

The experiments were performed in a completely randomized factorial design. The experimental data were statistically analyzed by using an ANOVA (analysis of variance) and a Tukey test with a 95% confidence level. Finally, Statgraphic software (Statgraphics Technologies, Inc., The Plains, VA, USA) was used for figures.

## 3. Results

### 3.1. Isolation and Identification of PGPB from Mature Compost

#### 3.1.1. Isolation of Bacteria Strain

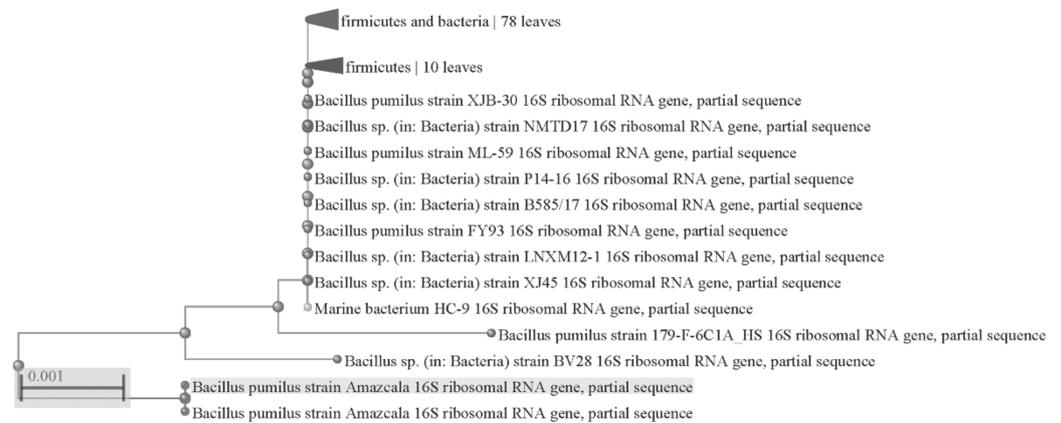
The bacterium isolated from the mature compost was a medium, circular, convex, whitish, and fusiform with rounded edges. When Gram staining was applied and observed under a microscope, it was determined that the bacterium was a Gram-positive bacillus.

#### 3.1.2. Molecular Characterization of Isolated Bacteria and Sequence Analysis

The phylogenetic tree constructed from 16S rRNA sequences showed that the selected isolate is a member of the genus *Bacillus*, and the sequence of the isolate showed 99% similarity to *Bacillus pumilus* strain MB411 in BLASTn analysis.

The phylogenetic position of *B. pumilus* was constructed by retrieving 16S rRNA gene sequences from closely related bacterial species using BLAST pairwise alignments at

<https://www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi> (accessed on 26 November 2021) (Figure 1).



**Figure 1.** Phylogenetic neighbor-binding tree reconstructed based on the 16S rRNA gene sequence showing the phylogenetic relationship between the isolated *B. pumilus* strain (MN067217) and similar strains.

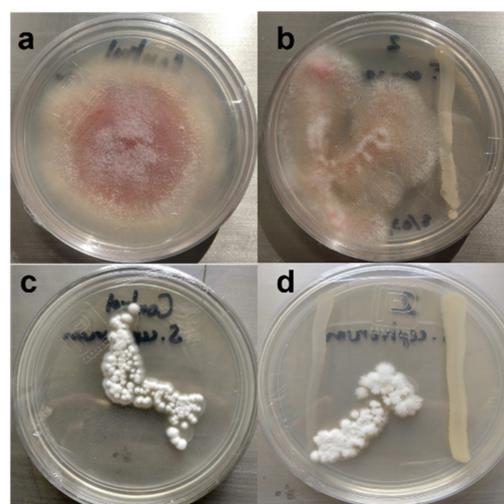
The partial sequence was deposited in NCBI GenBank with access number SUB5832921 Seq2 MN067217.

### 3.1.3. Phosphate Solubilizing Activity

*B. pumilus* showed a translucent halo in the Pikovskaya medium from the second day after inoculation. The bacterium had a phosphate solubilization index of 12.19 mm and a relative solubilization efficiency of 1119.23%.

### 3.1.4. Antifungal In Vitro Activity

The antifungal activity of *B. pumilus* against *Sclerotium cepivorum* and *Fusarium oxysporum* was evaluated using the direct antagonism test (Figure 2). In Table 1, it is observed that there is a representative statistical inhibition in *F. oxysporum* in presence of *B. pumilus*.



**Figure 2.** Antifungal in vitro activity of *Bacillus pumilus*. (a) *Fusarium oxysporum* control, (b) antifungal activity of *B. pumilus* against *Fusarium oxysporum*, (c) *Sclerotium cepivorum* control, (d) antifungal activity of *B. pumilus* against *Sclerotium cepivorum*.

**Table 1.** Diameter of the control fungus and the fungus in presence of *Bacillus pumilus*, and percentage of inhibition.

Fungus	Control Mycelium Diameter (cm)	Mycelium Diameter (cm) in the Presence of <i>B. pumilus</i>	% Inhibition
<i>Fusarium oxysporum</i>	7.267 ± 0.45 <sup>a</sup>	5 ± 0.87 <sup>b</sup>	31.19
<i>Sclerotium cepivorum</i>	3 ± 0.6 <sup>a</sup>	3.75 ± 0.35 <sup>a</sup>	−25.0

<sup>a,b</sup> Statistically significant differences between the Tukey treatments for  $p \leq 0.05$ .

*Bacillus pumilus* showed a 31.19% inhibition for *Fusarium oxysporum* and presented a statistically significant difference with the control. On the other hand, the bacteria promoted the growth of *Sclerotium cepivorum* by 25% without finding a significant statistical difference with the control.

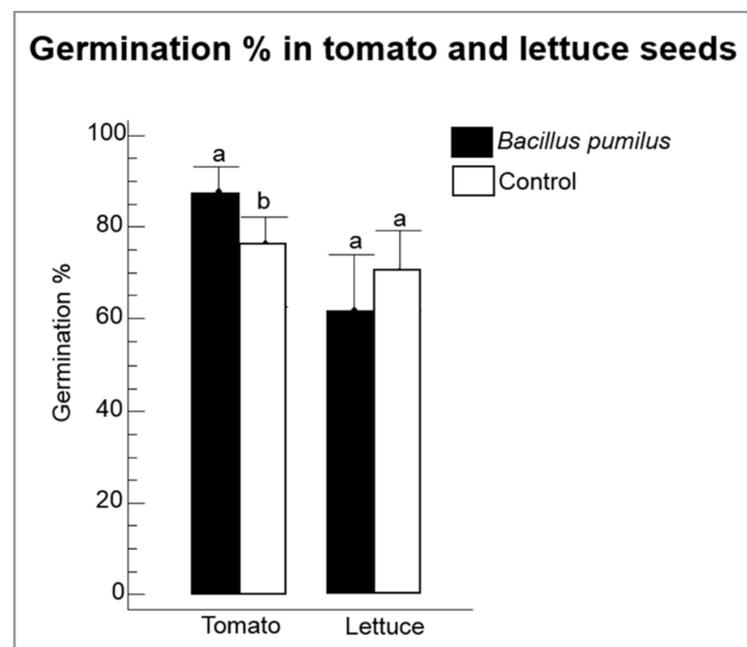
### 3.2. *Bacillus pumilus* Improves Plant Growth

#### 3.2.1. Effect of *Bacillus pumilus* Inoculation on Seed Germination

The effect of treatment with *B. pumilus* on germination varied according to the type of plant.

In tomato, *B. pumilus* increased germination by 11.12%, a statistically significant increase, obtaining a germination percentage of 87.65%, while the control without inoculation was 76.54%.

In lettuce, the seeds treated with *B. pumilus* had a germination percentage of 62.02%, while the control was 70.54%, without a statistically significant difference between both data (Figures 3 and 4).

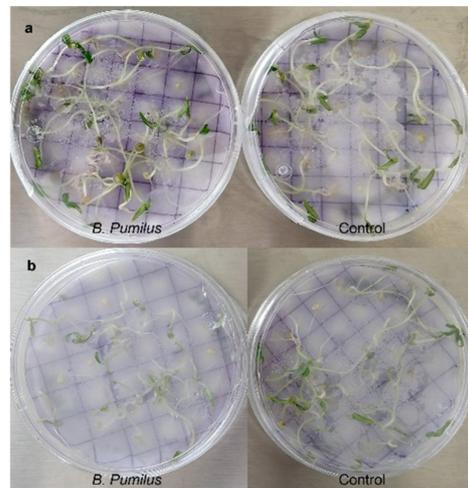


**Figure 3.** Effect of *Bacillus pumilus* on the germination of tomato and lettuce seeds. Error bars represent the standard deviation. The different letters indicate statistically significant differences between the Tukey treatments for  $p \leq 0.05$ .

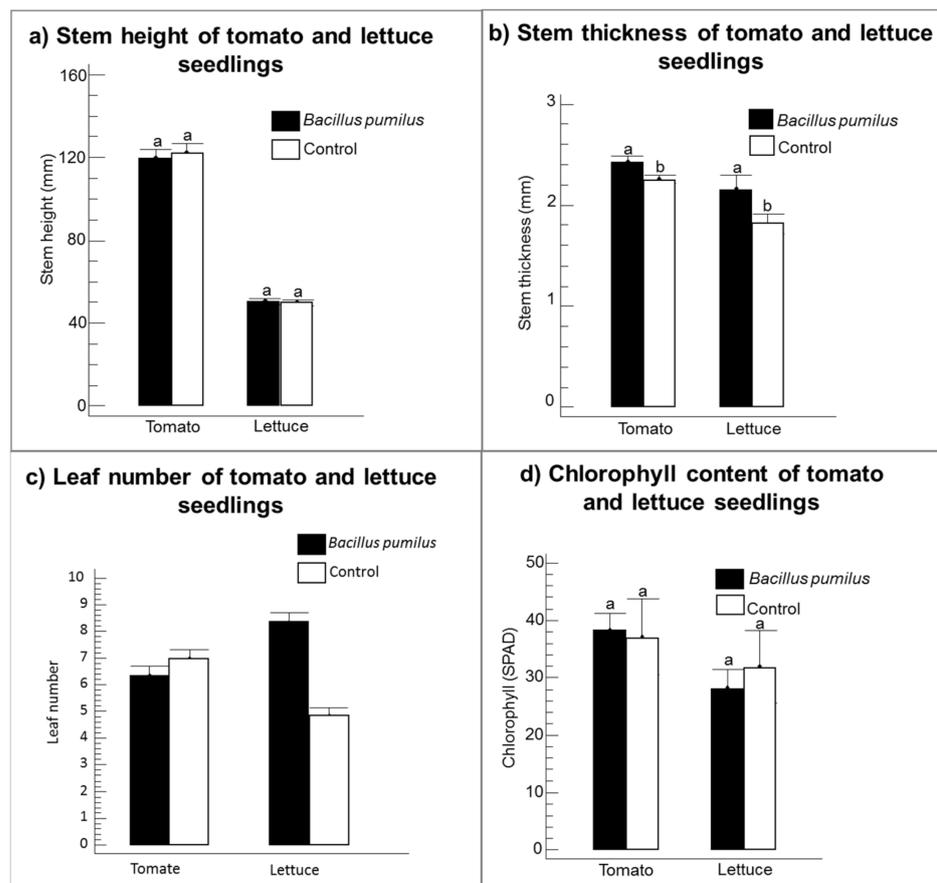
#### 3.2.2. Effect of *Bacillus pumilus* on Morphological Variables in Tomato and Lettuce Seed-Lings

The effect of *B. pumilus* on the growth of tomato and lettuce seedlings was evaluated. Height, stem thickness, leaf area, and chlorophyll content were measured and compared with an uninoculated control.

Inoculation with *B. pumilus* did not have a significant effect on the height of tomato and lettuce seedlings (Figure 5a).



**Figure 4.** Effect of *Bacillus pumilus* on the germination of tomato and lettuce seeds. (a) Tomato seeds germinated in the presence of *B. pumilus* and the uninoculated control. (b) Lettuce seeds germinated in the presence of *B. pumilus* and the uninoculated control.



**Figure 5.** Effect of *Bacillus pumilus* inoculation for 5 weeks on height (a), stem thickness (b), number of leaves (c), and chlorophyll content (d) in tomato and lettuce seedlings compared with a non-inoculated control. The error bars represent the standard deviation. The different letters indicate statistically significant differences between the Tukey treatments for  $p = 0.05$ .

On the other hand, the inoculation significantly increased the thickness of the tomato and lettuce stem. Figure 5b shows that tomato and lettuce seedlings had an increase of 7.64% and 18.87% in stem thickness, respectively. The stem thickness of tomato and lettuce seedlings increased with *B. pumilus* inoculation.

For the number of leaves, the inoculated lettuce seedlings had a significant increase, while in tomato seedlings they decreased—both data according to the uninoculated control (Figure 5c). Additionally, for the chlorophyll content, there was no significant difference between the seedlings inoculated with *B. pumilus* and the control plants (Figure 5d).

#### 4. Discussion

Many microorganisms are involved in the composting process, and some of them have the properties to promote plant growth [32]. The analysis presented shows how compost can be used as a source of biofertilizers, and the analysis allows a better understanding of biofertilizer use by characterizing a single microorganism of its microbiota.

The results of this study indicate that *Bacillus pumilus* isolated from mature compost has properties as a plant growth-promoter. The main characteristic of this bacterium is the solubilization of phosphate and the antimicrobial activity against *Fusarium oxysporum*. Therefore, *B. pumilus* can bring positive effects when used in agriculture. Yuan and Gao [33] reported that *B. pumilus* produces several bioactive substances: It has properties to promote plant growth and produces antimicrobial agents against bacteria and phytopathogenic fungi.

The isolated strain showed phosphate solubilizing activity, which may be due to the production of organic acids. These compounds are capable of mineralizing most of the organic sources of P present in the medium. The importance of PSB for agriculture lies in the fact that their application allows us to counteract the shortage of assimilable phosphorus and the increase in the cost of phosphorus fertilizers. Therefore, the inoculation of PSB in crops can reduce investment and loss of fertilizer through the efficient use of P [34].

In nature, some microorganisms can solubilize phosphates. In the *Bacillus* genus, we can find *Bacillus subtilis* with a PSI of 4.03 and RSE of 303.3% [35], *Bacillus cereus* with a PSI of 3.3 mm [36], and a *Bacillus pumilus* reported by Pandey [37] with a RSE of 175%. When we compare the previous data with the results obtained, we can observe a significant difference in phosphate solubilizing capacity. The data above indicate that isolated *B. pumilus* has potential qualities to be used as a plant growth promoter, providing phosphorus to plants [38] and possibly minimizing the use of chemical phosphate-based fertilizers.

Another aspect of the bacteria was its capacity to inhibit the growth of *F. oxysporum*. Heidarzadhe and Baghaee-Ravari [39] showed that *B. pumilus* minimizes the incidence of disease caused by *F. oxysporum* in vivo by 73%. Likewise, in the in vitro tests carried out by Rodríguez [40], a *B. pumilus* strain inhibited 63.87% *F. oxysporum* f. sp. cubense. The studies mentioned show similar results to those obtained in this study, but the effectiveness will depend on the bacterial strain used and the environmental conditions of the experiment.

Tomato and lettuce were used to evaluate the effect of *B. pumilus* inoculation on seeds and plants to determine how the bacteria act on each type of plant under the desired conditions [41].

The effect that *B. pumilus* had on the different types of seeds was contradictory. In tomatoes, it significantly increased germination, while in lettuce, it decreased overall germination. Previous studies show that the genus *Bacillus* can increase the germination percentage in different types of seeds. *B. megaterium* increases the germination of tomato seeds by 6.1% [42], while *B. circulans* does so by 14.56% [43]. Different *Bacillus* strains have been used to improve the germination of lettuce seeds. Malkoclu [44] found that a *B. subtilis* strain had a germination rate of 92%, and with a consortium between *B. subtilis* and *P. fluorescens*, the germination was 89.3%. However, there are bacterial strains such as *Azospirillum* sp., *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Pseudomonas cepacia* that, when are inoculated into lettuce seeds, decrease germination [45]. The information

above indicates that many factors influence seed germination, including plant hormones produced by soil bacteria [46].

Finally, the effect of *B. pumilus* in tomato and lettuce seedlings on height, stem thickness, leaf number, and chlorophyll content was measured.

The inoculation with *Bacillus pumilus* did not affect the height of the tomato and lettuce seedlings. It suggests that the isolate does not produce gibberellins. Previous studies show that the inoculation of *B. pumilus* and *B. amyloliquefaciens* does not have a significant effect on the height of tomato plants compared with a non-inoculated control. However, when adding a controlled-release fertilizer and the mixture of bacteria in soils with low nitrogen content, a positive effect on the height of the plants is observed [47]. The data suggest that the repercussion of PGPB on altitude is highly dependent on the type of crop and the strain.

The thickness of the stem of tomato and lettuce seedlings increased with *B. pumilus* inoculation. PGPB such as *Bacillus cereus* increase the thickness of tomato stem plants by 40.74% because bacteria release hormones that directly affect the tissue growth of vegetables [48]. In this study, the inoculation was functional for both vegetables—tomato and lettuce. However, we observed a higher effect in lettuce, suggesting that *B. pumilus*, similar to *B. cereus*, produces hormones that benefit plant tissue growth.

Finally, the leaf number in lettuce increased by more than 100% since *Bacillus pumilus* tends to accumulate some hormones, solubilizes phosphate, and produces large amounts of indole acetic acid that benefit plant growth [49].

The results of this study indicate that the isolated strain of mature compost, *Bacillus pumilus*, has properties as a plant growth-promoter. The main characteristic was the solubilization of phosphates. Therefore, the use of *B. pumilus* could be an efficient approach for improving crops and producing agriculture, combined with the reduction in P fertilizer, which has currently created a considerable dependence on current agricultural techniques of fertilization.

The strain of *B. pumilus* used in this study has not yet been evaluated for its capacity to improve plant growth in unsterilized soils and the field. However, the efficacy of the bacteria cannot be tested in other vegetables in addition to tomato and lettuce; for this, it is necessary to study and investigate how *B. pumilus* exerts its beneficial effects on the desired plants. Under the conditions evaluated, and due to its ability to solubilize phosphates, *B. pumilus* can be an efficient alternative for improving crops and minimizing the application of phosphate fertilizers. The latter is a current agricultural fertilization technique with considerable dependency.

## 5. Conclusions

It was possible to isolate and characterize bacteria with plant growth-promoting properties from a mature compost. The results of this study show that the inoculation of isolated *Bacillus pumilus* significantly improves the germination of tomato seeds and the growth of some physiological variables of lettuce and tomato seedlings, for which it is concluded that the isolated strain of *Bacillus pumilus* can be used as a plant growth-promoter in lettuce and tomato seedlings.

**Author Contributions:** Conceptualization, research, methodology, and writing—original draft preparation, B.M.-C.; resources and writing—original draft preparation, A.E.S.-G.; conceptualization, validation, and writing—review and editing, J.F.G.-T.; formal analysis and review and editing, M.T.-A.; conceptualization, validation, data curation, supervision, and writing—review and editing, G.M.S.-Z. All authors have read and agreed to the published version of the manuscript.

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