Article

Endospore-Forming Bacteria Present in a Commercial Stabilized Poultry Manure Determines the *Fusarium* Biocontrol and the Tomato Growth Promotion

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Received: 7 September 2020; Accepted: 14 October 2020; Published: 23 October 2020

Abstract: Stabilized organic amendments (SOA) from poultry are used in agriculture to improve the conditions of the soil. SOAs favor the growth of the crops and reduces the effect of soil-borne plant-pathogens. However, in northern Chile, there are no studies to support this observation, nor have the mechanisms involved in the beneficial effects observed in the field been established. This work aims to establish whether the promotion of growth and control of soil fungi in tomato observed in the field as a result of commercial SOA application can be attributed to different endospore-forming bacteria (EFB). The effect of commercial SOA on nutrient availability was determined. EFB isolated from a commercial product, and the application of bacterial isolates were compared with the commercial formulation of SOA, for plant growth promotion (PGP) and biocontrol of *Fusarium oxysporum* fsp. *radicis-lycopersici* (FORL). The local tomato cultivar *Poncho Negro* was used given its sensitivity to different nutritional alterations and FORL. A series of measurements of growth parameters were carried out in plants submitted to different mixtures of SOA treatments. Isolates were identified by biochemical tests and sequencing of the 16S rRNA gene. Eleven EFB were isolated from SOA, and some tests were performed to determine the PGP and biocontrol of FORL activities of each isolate. Notably, isolates BAC22 (*Bacillus megaterium*), BAC21, and BAC23 (*B. amyloliquefaciens/velezensis*) were associated with PGP, highlighting the ability to produce indole-3-acetic acid, a trait that in many cases is key to explaining the effects of *Bacillus* spp.

Keywords: PGP bacteria; poultry manure; biocontrol; *Fusarium oxysporum* fsp. *radicis-lycopersici*

1. Introduction

The soil of the Azapa Valley (Arica, Chile) is naturally poor in organic matter [1], and the cultivation of vegetables requires incorporating organic amendments in large quantities to achieve acceptable production levels. The stabilized organic amendments (SOA), generated from spent beds of the poultry industry, are mainly used in tomato production, and its incorporation allows the improvement of the biological conditions of the soil. The application of SOA, derivated from the poultry industry, favors the growth and mitigation of the effect of soil-borne plant pathogens, such as *Fusarium oxysporum* in tomato crops. However, there are no studies that support this observation, nor have the mechanisms involved in the effects observed in the field been established.

Different authors have shown that the use of manure as an amendment can replace the use of synthetic fertilizers, providing nutrients, improving soil structure, water retention capacity, porosity, as well as increasing the beneficial microbial population [2–6]. The microbial population present or induced as a result of the application of manure is largely responsible for the effects attributed to the

use of organic amendments [7], through an enhancement of nutrient availability, including carbon, nitrogen, and phosphorus, and improved soil pH, increasing microbial biomass, species richness and promoting the proliferation of certain species, which participate in the decomposition of complex organic matters and the promotion of plant growth [8].

The commercial SOA used in the Azapa Valley is produced through a standardized aerobic process that aims to eliminate phytopathogens, reduce odors, and decrease or eliminate potential putrefaction [9]. This process is maintained for more than two months, making sure that temperatures above 70 °C are produced for at least 30 days, decreasing the particle size considerably, and the C/N ratio drops below 35, allowing the marketing of this commercial SOA. This process favors the persistence of endospore-forming bacteria, which could play a key role in the beneficial effects on tomato plants by the application of SOA to the soil.

Among the endospore-forming bacteria that act as promoters of plant growth or plant growth-promoting rhizobacteria or PGPR [10], members of *Bacillus* genus are the most widely studied, being more than a half of the bioformulations of agricultural application produced from strains of *Bacillus* genus [11]. There are two main reasons for the success of this group of bacteria as bioformulates for agricultural use: (1) they can promote plant growth and phytopathogen control [12,13]; and (2) they possess the capacity to produce endospores and have a high degree of tolerance to different environmental challenges, including temperature, desiccation, ultraviolet light, among others [14,15].

In this work, we propose to establish whether the promotion of growth and control of soil fungus *Fusarium* in tomato observed in the field is a result of the application of commercial SOA to soil and could be attributed to the presence of different species of *Bacillus* genus and other endospore-forming bacteria. For this purpose, the effect of the application of commercial SOA on nutrient availability was determined. Subsequently, endospore-forming bacteria were isolated from the product, and finally, the application of bacterial isolates was compared with the commercial formulation of SOA, in the plant growth promotion and biocontrol of *Fusarium oxysporum* fsp. *radicis-lycopersici* (FORL).

2. Materials and Method

2.1. Plant Material

The local cultivar *Poncho Negro* was used, given its sensitivity to different nutritional alterations [16] and FORL [17]. The seeds were sown and grown for 20 days in the germination tray of 240 cavities in sterile peat before being used in the experiments.

2.2. FORL Strain Growth Conditions and Preparation

The strain of FORL F62 used in this work was isolated from a tomato crop in the Azapa Valley. This strain was identified and characterized by Sepulveda et al. [18] and maintained in the Phytopathogens Collection of the Plant Pathology Laboratory (University of Tarapacá). To ensure and re-activate its pathogenicity before being used for the experiments, it was inoculated into tomato plants as described below: for the infection of FORL in tomato, the fungus was grown for 7 days in Petri dishes with solid PDA (15% agar-agar). The mycelium was taken and planted in a flask with liquid PDA supplemented with 3% agar-agar and incubated in an orbital shaker incubator Gyromax SK727 (Amerex instrument, Concord, CA, USA) for 72 h at 150 rpm and 26 °C. A suspension of conidia adjusted to 1×10^6 conidia/mL was used to infect tomato plants.

2.3. Nutrients in Percolation and Tomato Growth with Different Doses of SOA

Poncho Negro seedlings were taken after 20 days of growth and placed in 250 mL plastic containers with 200 mL of different SOA/perlite mixtures: control corresponding to 200 mL of perlite (SOA 0% w/v); SOA 5% w/v, 10 g SOA/190 mL perlite; SOA 10% w/v, 20 g SOA/180 mL perlite; SOA 20% w/v, 40 g SOA/160 mL perlite; SOA 40% w/v, 80 g SOA/120 mL perlite; SOA 80% w/v, 160 g SOA/40 mL perlite. Aiming to know the effect of the different doses of SOA on the availability of nutrients, pH values, and

electrical conductivity (EC), after placing the plants in each of the respective treatments, holes were made in each plastic container to receive the percolation for 12 days. Table 1 shows the nutrients and the method used for each parameter analyzed.

Table 1. Organic matter, nitrogen content, acidity and electrical conductivity (EC) in stabilized organic amendment (SOA) samples.

Parameter	Unit	Observation *
EC 1:2.5	mS/cm	22.10
Acidity	pH	8.01
NO ₃ ⁻	mg/kg	246.00
Org. Mat.	%	32.65

* Each value represents the average of three repetitions.

2.4. Growth Evaluation of Tomato Plants

After 12 days, a series of measurements of growth parameters were carried out in the plants submitted to the different mixtures of SOA treatments. Plant height was measured from the neck of the plant to the last shoot, using a measuring tape. Leaf and root weight were determined using an analytical balance Adam NBL 84e (Adam equipment, Kingstone, UK). The number of leaves and length of leaves was registered. Additionally, the thickness of stems was measured using a digital caliper (Liaoning Mec Group Ltd., Liaoning, China).

2.5. Isolation of Endospore-Forming Bacteria (EFB) from SOA

Isolation of EFB from SOA was performed using the thermal shock method described by Al-Humam [19] with some modifications, 10 g of SOA was placed in 90 mL of sterile distilled water and agitated at 150 rpm for 1 h, and subsequently placed in a thermal bath at 80 °C for 30 min. After this step, the solution was maintained without agitation at room temperature for 2 h to sediment soil particles. Samples of 100 µL were taken from three different batches to perform three serial dilutions in Petri dishes containing solid Lysogeny Broth (LB) medium (ATCC medium 1065) and kept at 25 ± 1 °C. The isolated colonies with different morphologies were reseeded in plates with LB to obtain pure cultures of each isolate.

2.6. Biochemical Tests and Molecular Identification

Isolates were identified at the genus level using Gram staining, a catalase test, motility, starch hydrolysis, citrate utilization, endospore staining, and a hemolysis test [20]. For identification at the species level, the 16S rRNA gene was used. For this purpose, genomic DNA of each isolate was extracted according to Wang et al. [21], and the 16S rRNA gene was amplified with the forward primer 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer 1541R 5'-AAGGAGGTGATCCAGCCGCA-3' [22]. The PCR conditions consisted of 35 cycles at 95 °C for 2 min, 42 °C for 30 s, and 72 °C for 4 min. An additional final elongation step at 72 °C for 20 min was included. PCR products were purified using a peqGold gel extraction kit (PqLab, Erlangen, Germany). The purified fragments were sequenced by external services (Macrogen), and the resulting 16S rRNA gene sequences were compared to the GenBank database. Phylogenetic analysis was performed using MEGA X [23]. Sequences of each isolate were deposited in the GenBank.

The partial sequence of 16S rRNA obtained from each isolate and selected sequences retrieved from GenBank were aligned using Clustal W software [24]. The alignment was manually edited to obtain sequences of similar length (~1368 bp). Accession numbers of the retrieved sequences are indicated in parenthesis: *Bacillus megaterium* A-2 (MT026569.1), *Bacillus velezensis* IGM5-1 (MT197257.1), *Bacillus amyloliquefaciens* DST49 (MT176527.1), *Bacillus simplex* NBRC 15720 (NR042136.1), *Bacillus malikii* LM3308 (MF682944.1), *Bacillus thuringiensis* IAM 12077 (NR043403.1), *Bacillus mycoides* DSM 11821 (NR024697.1), *Bacillus subtilis* IAM 12118 (NR112116.2), *Bacillus thermoamylovorans* R-19047

(AJ586361.1), *Oceanobacillus* sp. AvH 7 (HQ316193.1), *Oceanobacillus sojiae* (AB473561.1), *Paucisalibacillus globulus* B22T (AM114102.1), *Sporosarcina thermotolerans* MER_59 (KT719638.1), *Sporosarcina globispora* 785 (NR029233.1), and *Sporosarcina ureae* DSM 2281 (NR041782). The phylogenetic tree was inferred using the neighbour-joining method with a bootstrap analysis of 1000 repetitions, and *Aquifex pyrophilus* Kol5a (NR029172.1) was used as outgroup.

2.7. Inhibition of FORL and Functional Traits Related Plant Growth Promotion in EFB Isolated from SOA

Eleven EFB strains isolated from SOA (routinely grown on LB medium) and the strain of FORL F62 (henceforth FORL) were used for dual culture tests. The dual culture assay was performed in 9 mm plates with solid PDA medium. A circle of 4 mm agar with grown FORL of 7 days was placed at the center of each plate, later 3 µL of each strain were inoculated separately in 6 points surrounding the circle of FORL and incubated for 7 days.

Different tests were carried out to determine the presence of traits related to the promotion of plant growth in the eleven EFB. The solubilization capacity of phosphorus was tested in solid Pikovskaya (PVK) medium, according to Majeed et al. [25]. Nitrogen fixation was determined using a semi-solid malate enrichment (NFb) medium, according to Döbereiner and Day [26]. The production of indoleacetic acid (IAA) was evaluated using the Salkowski reagent [27], and an IAA calibration curve was adjusted using an IAA standard (Sigma-Aldrich, St. Louis, MO, USA) using the following concentrations: 0, 3, 12, 18, 24, 40 and 50 µg/mL. Salkowski reagent (2 mL) was added at each concentration of the standard, and the absorbance at 530 nm was measured using a T60 UV/VIS spectrophotometer (PG instrument, Leicester, UK). The IAA produced by each isolate was measured in 4 day cultures grown in LB broth. The production of siderophores was evaluated using the chrome azurole S reagent (CAS) medium, according to Schwyn and Neilands [28], for the formation of halos after 5 days of incubation at 30 °C [29].

2.8. Promotion of Growth and Control of FORL in Tomato: SOA vs EFB

To compare the effect on growth promotion and biocontrol of FORL between SOA and the isolated EFB from SOA, a 10% v/v mixture of SOA and soil (20 mL SOA/180 mL soil) was used. To carry out the treatment with EFB, all the strains were grown separately in LB broth at 30 °C, and were subject to 150 rpm of agitation for 24 h in an orbital shaker incubator Gyromax SK727. Each isolated bacterium was diluted 1/10 in sterile water to a final volume of 110 mL. Seedlings were immersed for 1 h in the diluted solution and placed in the 250 mL plastic containers with the SOA 10% w/v mixture, depending on the treatment. SOA was autoclaved twice, at 120 °C and 1.5 kgf/cm² atm pressure, ensuring the substrate is completely sterile. Seven treatments were generated: T0 = control, plant placed in 10% w/v sterile SOA; T1 = plant pre-treated with the EFB and placed in 10% w/v sterile SOA; T2 = plant pre-treated with the EFB 24 h before infection with FORL and placed in 10% v/v sterile SOA; T3 = plants placed in 10% w/v SOA mixture without sterilization; T4 = plants infected with FORL and placed in 10% w/v SOA mixture without sterilization; T5 = plants infected with FORL and placed in 10% w/v sterile SOA; T6 = plants pre-treated with carbendazim (Goldazim 500 SC) (50 mL/100 L) 24 h before infection with FORL and placed 10% w/v sterile SOA. After 15 d, the severity was measured by a FORL severity index generated ad hoc (Figure 1), where 1 = healthy plant or with mild chlorosis, 2 = plant with chlorosis and wilting, 3 = plant with severe wilting, 4 = dead plant, and foliar weight and root weight were measured.

Figure 1. Representation of damage produced by *Fusarium oxysporum* fsp. *radicis-lycopersici* (FORL). This procedure was used to generate an ad hoc severity index.

2.9. Statistical Analysis

The data were subjected to analysis of normality and homogeneity of variance. ANOVA and Tukey test $p < 0.05$ were applied to determine statistical differences between treatments, for data that fulfilled the ANOVA assumptions. For non-parametric data, the Kruskal–Wallis test ($p < 0.05$) and a multiple comparison test of means present in the agricolae package for R [30] were used. All analyses were performed using the statistical software R.

3. Results

As an initial evaluation, some physical and chemical characteristics of SOA were analyzed, showing high levels of organic matter in SOA, but also high levels of EC and nitrates (Table 1), suggesting its application could generate toxicity in plants. To determine this toxic effect, an assay was carried out in tomato plants, in which different doses of SOA were used in a mixture with perlite, and a sample of the percolating irrigation was taken daily for chemical analysis. The mixtures of 20, 40 and 80% *w/v* SOA reached EC higher than 5 mS/cm (Table 2), which is not recommended for tomato cultivation [31], and a phytotoxic effect of these mixtures was expected. Mixtures of 40 and 80% *w/v* of SOA had the highest and fastest phytotoxic effect on tomato plants, and 24 h after the transplant, plants appeared withered and with severe saline damage, without recovery after 72 h. Mixtures of 10 and 20% *w/v* SOA presented attenuated damage, with the recovery of the plants after 72 h. The mixture of SOA 5% *w/v* showed some level of damage, but it was not permanent (data not shown). Since the doses above 40% *w/v* SOA affected the condition and survival of plants and are impracticable on the field, the evaluations with mixtures 0%, 5%, 10% and 20% *w/v* SOA were maintained (Figure 2; Table 3).

Table 2. Electrical conductivity and acidity in six mixtures of SOA: perlite mixture.

Mixture SOA: Perlite (% <i>w/v</i>)	EC (mS cm ⁻¹)	pH
0	0.26	6.82
5	2.53	7.75
10	4.78	7.69
20	7.89	7.66
40	18.38	7.30
80	25.50	7.25

Figure 2. Dynamics of nutrients in irrigation water percolated in different SOA: soil mixtures for 12 days. Blue line: control (SOA 0% w/v); purple line: SOA 5% w/v; green line: SOA 10% w/v; red line: SOA 20% w/v. The errors bars correspond to the standar deviation calculated on three repetitions. (a–h) indicate the physical-chemical parameters evaluated.

Table 3. Growth parameters of tomato plants grown in different mixtures of SOA:perlite. The values represent the mean $\pm$ standard deviation, calculated based on 15 repetitions. Different letters represent statistical differences according to the Tukey test ($p < 0.05$).

Mixture SOA/Perlite (% w/v)	Height (cm)	Leaf Number	Leaf Height (cm)	Foliar Weight (g)	Root Weight (g)
0	9.17 $\pm$ 1.61	3.33 $\pm$ 1.15a	6.98 $\pm$ 0.63a	1.49 $\pm$ 1.61a	1.47 $\pm$ 0.61a
5	13.33 $\pm$ 4.16	6.67 $\pm$ 0.58b	9.08 $\pm$ 1.89b	3.60 $\pm$ 0.16b	2.54 $\pm$ 0.16b
10	13.67 $\pm$ 3.21	5.67 $\pm$ 1.15b	10.14 $\pm$ 1.87b	4.08 $\pm$ 1.21b	2.82 $\pm$ 1.21b
20	15.33 $\pm$ 0.58	7.00 $\pm$ 0.00b	10.40 $\pm$ 1.09b	5.19 $\pm$ 0.58b	3.64 $\pm$ 0.58b

As observed in Figure 2, the first irrigation percolates show an important part of the nutrients are depleted in the soil solution of the treatments until reaching the levels of nutrients of the control (perlite) after seven days, except P_2O_5 , which reached it after nine days. The Electrical Conductivity (EC) had similar behavior in the soil solution and was decreasing with the days until reaching control levels. The pH did not experience significant changes regarding control with the different mixtures used, unlike what was observed in the other parameters.

There is a clear tendency of the growth of the plants treated with 5% w/v, 10% w/v, and 20% w/v SOA mixtures to increase (Table 3), which has also been observed on the field. Except for the height, statistical differences ($p < 0.05$) were established in all growth parameters between perlite and SOA mixtures, which was expected, taking into account the contribution of nutrients from SOA. There were no significant differences in the treatments with the three mixtures. However, 20% w/v SOA mixture produced a strong stress in comparison to 5%–10% w/v SOA. For this reason, subsequent experiments were performed using 10% w/v SOA mixture.

Eleven strains were isolated from SOA samples. Bacterial identification showed that ten isolates were classified as closely related species of *Bacillus* genus; meanwhile, one isolate was classified as non-*Bacillus* species. According to molecular affiliation and phylogenetic analysis of the 16S rRNA gene (Table 4, Figure 3), it was possible to identify each strain at the species level. Five strains were members of the *Bacillus* genus, three belonged to the *Paucisalibacillus* genus, two were related to the *Oceanobacillus* genus, and one strain was a member of the *Sporosarcina* genus. Particularly, strains BAC21 and BAC23 were classified as *B. amyloliquefaciens/velezensis* and BAC22 as *B. megaterium*, which are widely described as PGPR bacteria [32].

Table 4. Identification and in vitro plant growth-promoting traits of isolated strains from SOA. PS: phosphorous solubilization; NF: nitrogen fixation; SP: siderophore production; IP: indoleacetic acid (IAA) production. Symbol +: represents a positive reaction to the test; meanwhile, −: represents absent of the functional trait.

Bacterial ID			PS	NF	SP	IP
Strain	Affiliation	Accession Number				
BAC20	<i>Bacillus thermoamylovorans</i>	MT305775	+	+	−	+
BAC21	<i>Bacillus velezensis</i>	MT305776	−	+	−	+
BAC22	<i>Bacillus megaterium</i>	MT305777	−	−	−	−
BAC23	<i>Bacillus amyloliquefaciens</i>	MT305778	−	−	−	+
BAC25	<i>Oceanobacillus</i> sp.	MT305779	−	−	−	+
BAC26	<i>Paucisalibacillus globulus</i>	MT305780	−	+	−	+
BAC27	<i>Sporosarcina thermotolerans</i>	MT305781	−	+	−	−
BAC28	<i>Paucisalibacillus globulus</i>	MT305782	+	+	−	+
BAC29	<i>Paucisalibacillus globulus</i>	MT305783	+	−	−	−
BAC31	<i>Oceanobacillus</i> sp.	MT305784	+	−	−	−
BAC32	<i>Bacillus malikii</i> LM3308	MT305785	+	−	−	+

Figure 3. Comparative analysis of partial 16S rRNA gene sequences of the isolates obtained from SOA. The phylogenetic tree was inferred using the neighbor-joining method with a bootstrap analysis of 1000 repetitions.

Furthermore, a test of inhibition of FORL was performed in dual culture using each strain (Figure 4). The isolates BAC21, BAC22, and BAC23 showed an inhibitory effect above 50% of the growth of FORL, with 62.2%, 63.3% and 74.8% of inhibition, respectively. However, the other strains showed little or no inhibition of the growth of the fungus, standing out among these strains is the strain BAC20, which showed 20% growth inhibition of FORL.

Figure 4. In vitro biocontrol activity of isolated bacteria from SOA. Inhibition of FORL in dual culture. Each bar represents the percentage of three repetitions with its standard deviation. Under each strain, a representative figure of the fungal inhibition in the Petri dish is shown.

The presence of functional traits associated with the promotion of growth were identified to characterize plant growth promotion (PGP) activities (Table 4) functionally. Unlike the results obtained in the dual culture, in these tests BAC21, BAC22, and BAC23 strains did not show remarkable characteristics for the detected in vitro PGP traits. BAC20 strain had positive results for three of five functional traits, as well as BAC28. However, BAC28 produced very low levels of Indole Acetic Acid (IAA). BAC20 was also the one that showed IAA production followed by BAC21, and BAC23. In the case of the nitrogen fixation strains, BAC26, BAC27, and BAC28 had the most intense colorimetry concerning their fixation capacity. All this suggests a complementarity of the different strains regarding the PGP capacity.

To establish whether the EFB from the SOA product determines the plant promotion and FORL biocontrol in tomato, an experiment using tomato plants was performed. For this purpose, and based on the first experiment in plants, a 10% *v/v* SOA mixture for this procedure was selected. Figure 5 shows representative images of each treatment. It is possible to observe that the treatments T1, T2, and T3 showed more seedlings vigor with greater leaf area. T2 treatment (plants pretreated with the EFB in 10% *v/v* sterile SOA and infected with FORL) showed that the use of EFB helped to control the effects of FORL when it is compared to T5 treatment (plants were infected with FORL and placed in 10% *w/v* sterile SOA without EFB). In T2, the severity of FORL was significantly reduced ($p < 0.05$), which also had a significant growth increase, in both foliar and roots, regarding T5 ($p < 0.05$). Likewise, the pretreatment with the EFB allowed an equal phytopathogen control to that exercised by carbendazim, but with significantly higher growth of the roots ($p < 0.05$). T3 treatment (10% *w/v* without sterilization SOA) showed the same capacity to promote growth as the EFB applied to 10% *v/v* sterilized SOA, but this changed if FORL is included as was observed in T4 (Figure 5, Table 5) where FORL generated more damage by increasing the index of severity regarding T2 and having an index of severity statistically equal to T5. This greater severity of FORL in T4 also affected growth compared to the situation in T3, where it was not infected with FORL and more significantly with T2, where

the difference was higher concerning the growth and severity of FORL, and that can be attributed to pretreatment with the EFB.

Figure 5. Representative photograph of each treatment showing the growth and effects of FORL. T0 = control (10% *w/v* sterile SOA); T1 = plant pre-treated with the endospore-forming bacteria (EFB) and placed in 10% *w/v* sterile SOA; T2 = plant pre-treated with the EFB 24 h before infection with FORL and placed in 10% *w/v* sterile SOA; T3 = plants placed in 10% *w/v* mixture without sterilization SOA; T4 = plants infected with FORL and placed in 10% mixture without sterilization SOA; T5 = plants infected with FORL and placed in 10% sterile SOA; T6 = plants pre-treated with carbendazim (Goldazim 500 SCTM) (50 mL/100 L) 24 h before infection with FORL and placed in 10% sterile SOA. Red arrows indicate severity index 4. Blue arrows indicate severity index 3. Yellow arrows indicate severity index 2. Plants without arrows indicate severity index 1.

Table 5. Effect of the application of EFB isolated from SOA and the SOA product in the promotion of growth and biocontrol of FORL in tomato. The values represent the mean $\pm$ standard deviation, calculated based on 15 repetitions.

Treatment	Foliar Weight	Root Weight	Severity Index
T0	4.05 $\pm$ 0.23ab	1.13 $\pm$ 0.10bc	1 $\pm$ 0a
T1	4.45 $\pm$ 0.25a	1.75 $\pm$ 0.20ab	1 $\pm$ 0a
T2	4.49 $\pm$ 0.31a	1.8 $\pm$ 0.29a	1.3 $\pm$ 0.11b
T3	4.60 $\pm$ 0.28a	1.25 $\pm$ 0.11abc	1 $\pm$ 0a
T4	3.81 $\pm$ 0.29ab	1.19 $\pm$ 0.19bc	1.72 $\pm$ 0.24bc
T5	3.03 $\pm$ 0.33b	0.68 $\pm$ 0.04d	2.3 $\pm$ 0.31c
T6	4.24 $\pm$ 0.27ab	1.06 $\pm$ 0.09c	1.3 $\pm$ 0.3b

* Different letters in foliar weight and root weight represent statistical differences according to the Tukey test ($p < 0.05$). For severity index, different letters represent statistical differences according to multiple comparisons made after the Kruskal–Wallis test ($p < 0.05$).

4. Discussion

The application of manure and other biosolids allows the recycling of nutrients and the restoration of soil fertility [33]. However, the biological and physicochemical stability of these determine their effect on soil and plants. In a mature state of stabilization as it is in compost, it lowers the contribution of nutrients and improves the physical properties of the soil through the increase in the buffer capacity and the contribution of stability. In a state of medium maturation or stability, there are higher probabilities of problems associated with toxic concentrations of ammonium, nitrates, and soluble salts [34]. In this sense, the SOA used in this study with a medium state of stabilization presented problems of toxicity when concentrations are higher than 20% *w/v* in the soil mixture. Using mixtures less than or equal to 20% *w/v* SOA, a significant increase in growth is obtained. The phytotoxic effects observed with percentages of SOA < 20% *w/v* are reversible and transitory, and this is explained by the entrainment of nitrates, ammonium, and the decrease in the EC to perform the first three irrigations. According to our data, a concentration of less than 20% *w/v* SOA can be suggested, conducting at least three irrigations spaced throughout a day before placing the plants.

Although the plant growth promotion showed by the 5% *w/v*, 10% *w/v*, and 20% *w/v* SOA mixtures is partly attributable to the contribution of nutrients, the performance of the microbial flora cannot be ruled out. As previously mentioned, the SOA used in this work is marketed while there is still a dominance of the thermophilic stage. In this stage, there is a dominance of Firmicutes [35–37], allowing the focus to be on the search for growth promoter microorganisms related to endospore-forming bacteria (EFB) present in the commercial SOA.

It is recognized that there is a very low diversity compared to the works cited above, Huhe et al. [35]; de Gannes et al. [36]; and Takaku et al. [37], which used high-throughput sequencing. The low diversity obtained is attributed to the use of culture-dependent methods, but also to the salt characteristics that dominate the environment where poultry and SOA production takes place, which together with the filter associated with temperature, make this material even more astringent for the maintenance of different bacterial communities. In line with this, seven of the eleven isolated species belong to genera or species of Firmicutes associated with saline halophyte conditions, such as *Paucisalibacillus* sp., *Oceanobacillus* sp. *Sporosarcina* sp. This last genus has members such as *Sporosarcina ureae*, which develops in environments with high concentrations of urea. Moreover, BAC32 showed a high similarity to *B. malikii* (Figure 3), which was described as a heavy metal tolerant novel bacterium [38]. It is important to note that two isolates closely related to *B. amyloliquefaciens/velezensis* (BAC21 and BAC23) and one of *B. megaterium* (BAC22) were isolated, which have been widely described as PGPR and these strains could represent halotolerant isolates of these species.

The presence of trait associated with plant growth promotion (PGP) and the ability of direct antagonism on FORL in each bacterial isolate was determined. Regarding the trait associated with PGP, none of the strains achieved outstanding results in the evaluated traits (phosphorus solubilization, nitrogen fixation, siderophore production, and IAA production) highlighting mainly the ability to

produce IAA from BAC20 and BAC21 and BAC23, which is an important trait that in many cases plays a key role in the effects observed for *Bacillus* spp. [12]. From these analyses, it can be inferred that there may be a complementarity of the different strains concerning the PGP capacity. Regarding direct antagonism, only the *B. amyloliquefaciens/velezensis* (BAC21, BAC23) and the *B. megaterium* (BAC22) strains inhibited the mycelial growth of FORL by over 60%. Several authors have described the ability of this species to inhibit the mycelial growth of different phytopathogenic fungi, associating it with the capacity of this species of producing cyclic lipopeptides that act on the cell wall of fungi [39,40].

Finally, experiments using EFB present in the SOA product were able to promote plant growth and FORL biocontrol in tomato (Figure 5; Table 5). This experiment revealed that the EFB determine the control of FORL since in the cases where the sterile SOA was used together with the infection of FORL, there was no control, and the plants were severely affected by the fungus. Probably the most significant contribution to the biocontrol of FORL is performed by the two strains of *B. amyloliquefaciens/velezensis* and the strain of *B. megaterium*, which were the ones that achieved a satisfactory FORL mycelial inhibition in vitro. Previously, Baysal et al. [11] demonstrated the ability of different strains of *Bacillus* to inhibit mycelial growth and control FORL attacks in tomato plants. Regarding the PGP that is observed as a result of the application of SOA, it cannot be so clearly attributed to the bacteria since it was only possible to manage some parameters to establish statistical differences for the case of root growth between plants with sterile SOA application and with the presence of EFB. It is likely that with a longer-term experiment, these differences are also expressed in higher leaf growth, given the greater ability to absorb nutrients of plants with a robust root system.

In conclusion, the application of EFB members of *Bacillus* genus isolated from a commercial SOA has a beneficial effect on plant growth and FORL biocontrol in tomato plants, revealing an enormous potential for the development of new strategies that favor higher horticultural production of tomato with reduced agrochemical loads and the production of innocuous tomato.

Author Contributions: All authors contributed to the study conception and design. Material preparation, sampling, and experimental procedures were performed by P.M.T., M.A.M., and G.F.S.C. Data analysis was executed by P.M.T. and G.F.S.C. The first draft of the manuscript was written by G.F.S.C. and revised by P.M.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Ministry of Education, Government of Chile Convenio Desempeño en Educación Superior—U. Tarapacá 1795 (Arica-Chile).

Conflicts of Interest: The authors declare no conflict of interest.

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