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Microbial endophytes that live within seeds of two Tomato hybrid cultivated in Argentina

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Abstract:

Tomato (*Solanum lycopersicum* L.) is widely consumed around the world is mostly affected by stresses and diseases that reduce yield and production. Research on sustainable technologies like the use of beneficial microorganismsis crucial to development sustainable management strategies. Endophytic bacteria might increase production as well as plant health.. In this work we studied the endobiome of tomato seeds of different cultivars since the plant genotype might affect the microbial community structure in terms of plant growth promoters as well as organisms for biocontrol. The conditions prevailing within seeds along the maturation period might have affected bacterial survival. This is such that seed endophytes share features, which are different from those of bacteria from other plant tissues. The community associated with different cultivars reflects the different resources available in the seed and its potential to prevent the attack of pathogens and to promote plant growth.

Keywords: ENDOPHYTES; TOMATO; BIOCONTROL; PGPR; SEED, MICROBIOME.

1. Introduction

Tomato (*Solanum lycopersicum* L.) is among a vegetable that, based on its production and consumption, is of the most important along the world (http://faostat.fao.org). Like other plants, their genome is complemented by a pletophora of genes provided by organisms that an associated to their surfaces as well as intracellular spaces that is now known as the phytobiome [1-3]. This mountable of genes and proteins provided by these organisms is such that they are considered as members of the plant genomes, since they definitely impact on fruit quality and yield of different varieties [4].

Recently, research on cleaner and sustainable technologies alternative to chemical fertilizers and pesticides has gained importance. These technologies tend attempt to use bacterial communities associated with plants, known as the microbiomes, are potential sources to select organisms or groups of them to develop products to promote plant growth and/or protect plants against stresses including pathogens [5, 6]. In past decades, tens of thousands of plant-associated bacterial have been isolated and have been shown to promote plant growth or control plant pathogens [4-6]. The most predominant and best-studied microorganisms that have been isolated from plant tissues belong to three major phyla, Actinobacteria, Proteobacteria and Firmicutes, including members of the genera *Streptomyces*, *Pseudomonas*, *Azoarcus*, *Enterobacter*, *Burkholderia*, *Stenotrophomonas* and *Bacillus*, among others [7].

The diverse array of microbial communities within tissues of different plants organs have been defined as endophytes [1]. It is widely accepted that these organisms are ubiquitous colonizers of plants and, therefore, influence plant health and productivity [8]. Endophytes may benefit hosts

through diverse mechanisms, such as molecules that increase their capacity to compete for space, nutrients and/or ecological niches; the synthesis of antimicrobial substance or the synthesis of inducers of plant growth or compounds like phytohormones and peptides that might keep vegetables or plant organs healthy, which additionally might have no negative effects on consumers and/or the environment [9-14].

The main source of endophytes might be seeds provided organisms can be transmitted once the sexual reproduction occurred. To do this, organisms should be able to move within the plant and survive within the seeds that have low water content. Probably, most seeds carry a diverse array of endophytes and this is not surprising considering that seeds represent a remarkable phase in the life cycle of spermatophytes. Endophytic organisms can persist for years in a seed under a dormant state and whenever environmental conditions are amenable for seed growth, a new plant develops carrying the surviving organisms [15]. Seed endophytes are transmitted from generation to generation, which means that along evolution the microbiome of plants might became indispensable to complete their life cycle. This vertical transmission should select against pathogenicity and favor mutualism as these endosymbionts depend on their host for survival and reproduction but also might be beneficial for plants [16, 17]. Because of this, it is critical to know which are the bacteria communities associated with plants and how are such populations affecting plants regarding their growth, health and survival ability under stressful environments. Interestingly, there are not very many studies looking at bacteria associated with seeds compared to research on rhizospheric bacteria [18, 12]. Xu et al. [19] isolated 84 culturable endophytic bacteria from tomato seeds of different varieties, and demonstrated that the endophytic bacterial community structure is a function of each variety. The *16S rDNA* PCR-RFLP analysis revealed that tomato seeds contained an endophytic community of bacteria quite diverse. Interestingly, all isolated bacteria belonged to *Bacillus*, an endospore-forming genus of Firmicutes, which could be related to their ability to survive under dehydration and starvation since they form endospore [20, 12, 21]. The conditions prevailing within seeds along the maturation period varies along the process and this might affect bacterial survival within seeds. Seed endophytes share some characteristics that might not be typical by endophytes from other plant tissues [12, 21]. Truyens et al. [21] analyzed several studies on seed endophytes and highlighted that bacteria found in seeds of many different plants mostly belong to *Bacillus* and *Pseudomonas*, and at lower frequently to *Paenibacillus*, *Micrococcus*, *Staphylococcus*, *Pantoea* and *Acinetobacter*. In any case these bacteria all differ in their strategy of survival.

Recently, community analysis of culturable and unculturable microorganisms interacting with plants was performed by means of new generation sequencing technologies. In such studies the phytobiome of tomato and sugarcane roots [22] were formed mainly by Actinobacteria, followed by Proteobacteria, Bacteroidetes and Firmicutes [23, 8]. It is interesting to highlight that, Streptomycetales and Pseudomonadales were found to be highly enriched and are therefore the predominant organisms within tomato roots. Furthermore Micromonosporales, Rhizobiales, Sphingomonadales, Burkholderiales, Xanthomonadales and Flavobacteriales also were among the most abundant bacterial groups [22].

Culture-dependent experiments provided an enormous amount of information regarding the beneficial effect of endophytic isolates [4, 8, 13], which was also confirmed when High-throughput sequencing-based metagenomic and genomic studies. These studies provided information regarding the structure of these microbial communities and the ability of these organisms to adapt to different environments [24, 25].

The purpose of this work was to study the phytobiome of tomato seeds of different hybrids in order to know the importance of the plant genotype on the community structure in terms of organisms with PGPB potential to promote growth and control plant pathogens.

2. Materials and Methods

Bacterial community structure and diversity.

Endophytic bacterial DNA of tomato were isolated from seeds of 2 cultivars Elpida F1 (Enza Zaden) and Silverio (Syngenta-Rogers). Isolation of DNA from seed samples was performed on

three replicates, each consisting of 20 seeds. First, seeds were surface disinfected in 5 % commercial bleach and 0.01 % Tween 20 for 10 min and rinsed with sterile distilled water. In order to check the superficial sterilization of seed, the water used for the final wash was plated on tryptic soy agar (tryptone, 17.0 $g.l^{-1}$; soytone, 3.0 $g.l^{-1}$; NaCl, 5.0 $g.l^{-1}$; K_2HPO_4, 2.5 $g.l^{-1}$; glucose, 2.5 $g.l^{-1}$; agar, 20.0 $g.l^{-1}$). Furthermore, aliquots of this water were included in PCR reactions used at amplifying the *16S rDNA* gene.

Seeds of each cultivar were homogenized in 0.95 % (w/v) NaCl, and the extract was filtered through filter paper to separate bacterial cells from seed debris. The filtrate was centrifuged (10 min; 15 000 x g), and the pellet was used as the source for the extraction of genomic DNA, which was performed with the commercial kit Wizard® Genomic DNA purification Kit (Promega) [26].

The *16S rDNA* gene V1-V3 region was amplified using 27F (5'-AGRGTTTGATCMTGGCTCAG-3') [27] and 519R (5'-GTNTTACNGCGGCKGCTG-3') primers [28], with barcode on the forward primer for MiSeq instrument (Illumina Inc., San Diego, CA). PCR was performing using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94 °C for 3 minutes, followed by 28 cycles of 94 °C for 30 seconds, 53 °C for 40 seconds and 72 °C for 1 minute, after which a final elongation step at 72 °C for 5 minutes. After amplification, PCR products are checked in 2 % (w/v) agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples are purified using calibrated Ampure XP beads. Then the pooled and purified PCR product was used to prepare illumina DNA library. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines.

Mothur pipeline was used for the entire sequence data processing according to the Mothur SOP [29]. Error were removed through screening sequences that did not align to Silva database (nr v119) [30], preclustering to merge rare sequences into larger sequences if the difference is within one or two base pairs, according to procedure described by Allen and co-workers [31]. Chimeras were removed by using uchime (UCHIME) [32]. Taxonomic classification was assigned by aligning to mothur's implementation of the SILVA database, followed by non-bacterial sequence removal. Singletons sequences were removed (defined as sequences that occurred only once among all samples). The final sequence data were then clustered into Operational Taxonomic Units (OTUs) split by 3 % genetic distance using the average neighbour method. Hill numbers, ${}^0H$ (richness), ${}^1H$ (diversity) and ${}^2H$ (equitability) were used to compare bacterial alpha diversity [33, 34] and were calculated using Mothur software [29].

Isolation of bacteria from tomato seeds

Endophytic bacteria of tomato were isolated from seeds and seedling of 2 cultivars Elpida F1 (Enza Zaden) and Silverio (Syngenta-Rogers) by culturing them on three different commercial culture media (TSA, Nutritive agar and King B - BritaniaLab S.A.).

Seeds were surface sterilized as described above. The effect of the sterilization procedure was confirmed by placing sterilized seeds on culture media. In order to generate seedling seeds were superficially sterilized and were seeded in glass tubes (25 cm high and 3 cm in diameter) containing semisolid Hoagland solution (8 $g.l^{-1}$ agar). The tubes were incubated at 30 ° C with a photoperiod of 16 hours for 30 days. At this time, tomato seedlings were harvested and surface sterilized as described above. The sterile seeds and seedling of each tomato cultivar were crushed and homogenized in 3 ml of 3 strength Ringers solution (215 mg of NaCl, 7.5 mg of KCl, 12 mg of $CaCl_2$ $2(H_2O)$, 50 mg of $Na_2S_2O_3$ $5(H_2O)$ in 100 ml of distilled water, pH adjusted to 6.6) and aliquots of the supernatant were plated on the three media and plates were incubated at 28 °C for 5 days [35]. After a 5 day incubation period colonies developed and were morphologically characterized in terms of size, shape and color and were sub-cultured until pure cultures were obtained. Then isolated bacteria were grown in liquid media until saturation and aliquots were mixed to make a final concentration of 10 % glycerol, tubes were kept at −80 °C.

Extraction of genomic DNA, PCR amplification and sequencing of *16S rDNA* gene.

DNA was extracted from bacterial isolates using the Wizard® Genomic DNA Purification Kit (Promega). Isolated bacteria were cultured in liquid media until their cell concentration was approximately 1 x 10^9 cells.ml^{-1} aliquots of these cultures were extracted by following the procedure recommended by the manufacturer. The quality and quantity of the isolated DNA was checked by electrophoresis in 7 % agarose gels stained with ethidium bromide that included a control sample of known concentration.

In order to characterize the organisms further they were fingerprinted by means of BOX-PCR using the universal BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG- 3') [36]. PCR amplification and electrophoretic analysis were carried out as described in López and Balatti [36]. We selected for further analysis all those bacterial cultures that presented a different fingerprint. The identity of these organisms was initially analyzed by means of the 1,500 bp sequence coding for the *16S rDNA*. Such fragments were amplified by PCR in a thermocycler (MinicyclerTM – MJ Research), by means of primers 27F and 1492R [27]. PCR products were purified and sequenced. The *16S rDNA* gene sequences determined in this study, have been deposited in the GenBank database under accession numbers MG963203 to MG963224.

Sequence analysis and alignment were performed with 16S biodiversity tool Geneious R9 software. Species classification using *16S rDNA* amplicon sequencing data from bacterial samples were performed using the cloud-based *16S rDNA* biodiversity tool (Geneious version R9.0.5, Biomatters, http://www.geneious.com) [37].

***In vitro* antagonism of bacterial isolates towards tomato pathogens**

***In vivo* bioassays of pathogens inhibition effects of bacteria**

Bacterial isolates were cultured as previously described. The pathogens *Alternaria alternata*, *Corynespora cassiicola* and *Stemphylium lycopersici* (strains CIDEFI 209, CIDEFI 235, CIDEFI 234, respectively) were cultured on APG (BritaniaLab S.A.).

In vivo antagonism bioassays were carried out to evaluate inhibitory effects of 41 endophytic bacterial isolated from tomato seeds on pathogens growth. Bacterial strias were made on nutritive agar plates that were divided in three sections, in which different fungal isolates were plated. Simultaneously, 5 mm mycelial plugs cut from the edge of seven day-old culture of the fungal strain were placed at the quadrant centre of the plate. All the plates were incubated at 25 °C for 5 days and examined for evidence that growth of the fungus was inhibited by the bacterium. A positive response was the visible zone of inhibition around the fungus.

Inhibitory activity of the cell-free supernatant of endophytic bacteria against fungi pathogens.

Five selected bacteria (E4, E7, E9, S15 and SE37) and Er-S (*Bacillus subtilis*) as control were cultured in liquid nutrient broth in a rotator at 180 rev.min^{-1} at 28 °C in the dark for 48 h. The cell-free cultured supernatant was collected by centrifugation at 6000 x g for 15 min, and sequentially filtered through 0.45 µm and 0.22 µm organic filter membranes (©GVS). The antimicrobial activity of culture filtrates were evaluated against pathogens such as *A. alternata*, *C. cassiicola* and *S. lycopersici* that were cultured as described previously.

The inhibitory activity against mycelial growth of the cell-free supernatant was measured by adding extracts to agar plates (1.5 % w/v agar) containing nutritive agar to make a final concentration 1 %, 10 % and 20 % (v/v). Then, a 5 mm mycelial plug was removed from the margin of the fungal colony and placed in the quadrant centre of the plate. Plates were incubated for 4 days at 25 °C and fungal growth was measured. The inhibitions activity was expressed in terms of percentage of mycelial growth inhibition and was calculated according to the following formula: Inhibition (%) = [(Growth in control – Growth in treatment)/Growth in control]*100 [38].

Effect of volatiles from endophytic bacteria against fungal pathogens.

A bioassay was performed in sealed dishes using the method described by Baysal et al. [38], with some modifications. Briefly, 300 µl of bacteria cultures were spread onto a sterile plate containing TYB medium (g l^{-1}) (tryptone 10, yeast extract 5, beef extract 3, glucose 20, KH_2PO_4 0.5, Mg_2SO_4 0.3, $MnSO_4$ 0.07, Fe_2SO_4, citric acid 0.3. agar 1.5, pH 7.2). Five mm fungal mycelial plugs taken from the margin of the colony were then placed in the centre of another plate containing PDA

[39]. The fungal dishes were immediately inverted over and placed on taps of the plate with bacterial culture and were rapidly sealed with parafilm. Plates were incubated at 25 °C in the dark until the fungal mycelium of the controls extended to ¾ of the plate. Control was mounted with plates containing only TYB medium. The diameter (mm) of the fungal colony was measured.

Bacterial effect upon tomato growth.

Tomato cv Elpida were growth in vitro in culture media that contained in 4.4 $g.l^{-1}$ of MS Basal Salts with Minimal Organics, 15 $g.l^{-1}$ of sucrose, 7.5 $g.l^{-1}$ of agar, and a pH of 6. Ten ml of cultured were poured into culture tubes that were tomato seeds were seeded: were inoculated with a bacterial suspension. This was made by resuspension of bacterial colonies in 50 mM Na_2HPO_4 (pH 7) to an OD_{600} of 0.2, and 100 ml injected onto each tube. Each treatment had three replicates that were incubated for 30 days in a growth chamber with 50 % humidity and 16-hour photoperiod. Then plants were remove from de tubes and were cleaned. Roots and shoots were placed in oven until constant weights and recorded dry weights compared to the mean of control tubes without inoculate.

Siderophore and phytohormone production and phosphate solubilization

Siderophores production was tested qualitatively using chrome azurol S (CAS) agar as described by Alexander and Zuberer [40]. CAS agar was made by means of three solutions that were prepared and sterilized separately and then mixed. The 10 ml Fe-CAS indicator solution (solution 1) was made up of 1 mM $FeCl_3$ $6(H_2O)$ dissolved in 10 mM HCl, 50 ml of an aqueous solution of CAS (1.21 $mg.ml^{-1}$), and 40 ml of an aqueous solution of hexadecyl-trimetylammonium bromide (HDTMA) (1.82 $mg.ml^{-1}$). Solution 2 (buffer solution) was prepared by dissolving 30.24 g of PIPES (piperazine-N,N′-bis[2-ethanesulfonic acid]) in 750 ml of salt solution, distilled water was added to bring the volume to 800 ml. Once the pH was adjusted to 6.8 with 50 % KOH, 15 g of agar were added, and the solution was autoclaved. Solution 3 contained 2 g glucose, 2 g mannitol and trace elements in 70 ml of distilled water, when we mixed solution 1 the color changed to a dark green. Siderophores-production was determined by the appearance of an orange halo around colonies after an incubation period of 24 h. Tree replicates of bacteria were cultured on CAS agar plates. Phosphate solubilization was determined as described by Castagno et al. [41]. Bacterial strains (16-h-old cultures) were spotted on plates containing National Botanical Research Institute phosphate growth medium (NBRIP) (5 g l^{-1} $MgCl_2$ $6(H_2O)$, 0.25 g l^{-1} $MgSO_4$ $7(H_2O)$, 0.2 g l^{-1} KCl, 0.1 g l-1 $(NH_4)_2SO_4$, 5 g l^{-1} $Ca_3(PO_4)_2$ and 10 g l^{-1} glucose) and incubated at 28 °C for 24-48 h. Phosphate solubilization was evidenced by the development of a clear halo around the colony.

Phytohormone production was evaluated on agar plates (9-cm diameter) inoculated with toothpicks into a grid pattern within agar cultures. Grid plates consisted of replicate rows of several isolates per plate. Each inoculated plate was overlaid with an 82-mm-diameter disk of nitrocellulose membrane (Amersham). All plates were incubated until colonies reached 0.5 to 2 mm in diameter. After an appropriate incubation period 24-48 h, the membrane or paper was removed from the plate and treated with Salkowski reagent that was 2 % 0.5 M $FeCl_3$ in 35 % perchloric acid. Membranes were saturated in a Petri dish by soaking directly in reagent [42].

Biofilm and autoagregation assays

Bacteria were grown in 2 ml nutritive broth, incubated for 24 h at 28 °C, diluted 1/100 in nutritive broth and incubated 48 h under the same conditions. Bacterial suspensions (5 ml) were then transferred into a glass tube (10 by 70 mm) and allowed to settle for 24 h at 4 °C. A 0.2 ml aliquot of the upper portion of the suspension was transferred onto a microliter plate and the final optical density at 630 nm (OD_{630nm}) (OD_{final}) was measured. A control tube was vortexed for 30 s and the initial OD_{630nm} ($OD_{initial}$) was determined. The percentage of autoaggregation was calculated as follows: $100 * [1-(OD_{final}/OD_{initial})]$ [43].

Biofilm formation was determined macroscopically by a quantitative assay with 96-well microtiter dishes, whereby biofilms were stained with crystal violet (CV) as described by O'Toole and Kolter [44], with modifications [43]. Bacteria were grown in 2 ml nutritive broth and incubated with agitation for 48 h at 28 °C. Cultures were diluted with fresh medium to give an OD_{630nm} of 0.1. One hundred microliters of the bacterial suspension was added to each well and incubated with

agitation for 24 h at 28 °C. Bacterial growth was quantified by measuring the OD_{630nm}. Cells were gently removed, 180 µl CV aqueous solution (0.1 %, w/v) was added and stained proceeded for 15 min. Each CV-stained well was rinsed thoroughly and repeatedly with water and then scored for biofilm formation by adding 150 µl 95 % ethanol. The OD_{560nm} of solubilized CV was measured with a MicroELISA Auto Reader (KartellTM – Fisher Scientific). In parallel, sterile control cultures were made with nutritive broth.

Autoaggregation assays were performed six times. In the biofilm assays, each strain was plated onto at least 12 wells of each microtiter dish. The data were subjected to a one-way analysis of variance (ANOVA), followed by a comparison of multiple treatment levels with the control by using Tukey test. All statistical analyses were performed by using Infostat, version 1.0.

3. Results

3.1. Bacterial community.

3.1.1. Total bacterial community structure and diversity

The results confirmed that the disinfection procedure was effective in eliminating both cultivable and non cultivable epiphytic bacteria, as well as potential DNA traces from the seed surface.

The V1-V3 region of the *16S rDNA* gene of two biological replicates of a seed DNA from two cultivars of tomato (Elpida and Silverio) on the MiSeq platform was sequenced. Sequence data used in this study were deposited to the NCBI Sequence Read Archive (SRA, www.ncbi.nlm.nih.gov/sra) and are available with the accession number PRJNA438294.

Table 1 shows the result of amplicon sequencing analysis. Sequencing data analysis and subsequent statistical inference from the samples provided up to 362,180 sequences, which resulted in 47,323 useful *16S rDNA* sequences after the trimming process. The number of sequences of all treatments was normalized to the smallest number of observed sequences obtained from Elpida seed which was 10,254. The Good´s coverage of the prokaryotic diversity was greater than 86 %, for trimmed and normalized data from all the systems. The diversity and richness indexes [33] in the studied samples studied suggested that Silverio seed had a bacterial community with a larger richness (^0H) than Elpida seed, although with similar species diversity (^1H). Also, in both communities; the most common species were present in a slight prevalence (^2H), resulting an unequal assemblage of the community. Figure 1 and 2 shows taxonomic profiles of the bacterial community in each system at phylum and order level with the relative abundance (> 0.5 %). The orders with relative abundance < 0.5 %, were grouped in “Others”.

Seed endophytic bacteria of both tomato cultivars were mainly represented by four phyla (Figure 1). In this regard, Firmicutes made up 50 % of the endophytic community of Elpida seeds, followed by Proteobacteria, (28 %), Actinobacteria (20 %) and also included a small proportion of Bacteroidetes (2 %). The latter one was also reported as the smallest bacterial community component on Silverio seeds. Proteobacteria (45 %) and Actinobacteria (48 %) were the main components of the endophytoc community of Silverio seeds, while Firmicutes a small represented (5 %) of this community (Figure 1). Among Proteobacteria, Gammaproteobacteria was the most abundant class in tomato Elpida and Silverio, 82 and 66 %, respectively of endophytic community. Alphaproteobacteria and Betaproteobacteria only represented 15 and 3 % of endophytic bacteria in Elpida and 27 and 7 % in Silverio seeds samples.

The composition of the endophytic communities of Elpida and Silverio were significant different. While Actinomycetales (14.3 %), Bacillales (63.3 %) and Psedomonadales (14.6 %) were de most abundant ones in Elpida in cultivar Silverio there most important were Actinomycetales (27.3 %), Rhizobiales (16 %) and Pseudomonadales (37.3 %) (Table 2). However, the composition of genus of these Order were similar for both samples. In both samples, Actinomycetales included mainly the genus *Clavibacter*, *Corynebacterium*, *Micrococcus*, *Curtobacterium* and *Microbacterium*.

Pseudomonadales was found to contain OTUs assigned to the genus *Pseudomonas*, *Moraxella* and *Acinetobacter*; several others OTUs assigned to Bacillales and Rhizobiales were classified at the genus *Paenibacillus*, *Staphylococcus*, *Shinella* and *Sphingobium* (Table 2).

3.1.2. Culturable bacterial community

A total of 41 isolates obtained from seed and tomato seedlings, a subset of 21 of them were analyzed. According to their BOX-PCR profiles, the strains: E4, E6, E7, E8, E9, S15, S19, S20, S21, S26, S27, SE28, SE31, SE33, SE34, SE35, SE36, SE37, SS38, SS39 and SS41 were considered to be unique among isolates.

The taxonomic identity of 21 isolates was assessed by comparing *16S rDNA* sequences with these of references strains available at the Gene Bank database. The results are presented in Table 3 and were consistent with clustering evidenced by 16S biodiversity graph (Figure 2), generated with 16S Biodiversity tools of Geneious software (Geneious version R9.0.5, Biomatters, http://www.geneious.com). This analysis showed that Firmicutes were the most abundant class of microbes observed within the materials used in this study, being *Bacillus* and *Paenibacillus* the most common genera. Also represented were the classes Alpha-Proteobacteria, Gamma-Proteobacteria and Actinobacteria (Figure 2).

Elpida seeds contained mainly Firmicutes 80 % and Actinobacteria 20 % and from seedlings of this cultivar we isolated Firmicutes 72 %, Actinobacteria and Gamma-Proteobacteria 14 % each. The Actinobacteria found belong to the genera *Micrococcus* and *Microbacterium* and among Gamma-Proteobacteria we isolated a number of the genus *Acinetobacter*.

When the sources of isolation were Silverio seeds again we isolated mostly Firmicutes (67%) and among Alpha-Proteobacteria (33 %) two different genera (*Sphingomonas* and *Brevundimonas*); whereas bacteria isolated from seedlings of this cultivar included only Firmicutes (100 %). Among Firmicutes isolated from seeds and seedlings of both cultivars bacteria belong to the following genera *Bacillus*, *Paenibacillus*, *Psychrobacillus* and *Jeotgalibacillus*.

After identifying by the *16S rDNA* sequence, their ability to promote growth and to antagonize *A. alternata*, *C. cassiicola* and *S. lycopersici*, were evaluated.

3.2. *In vitro antagonism of bacterial isolates towards tomato pathogens*

3.2.1. *In vivo* antagonism of bacterial isolates towards tomato pathogens

The biocontrol potential of the 21 isolates was tested in Petri plates where fungal pathogens such as *A. alternata*, *C. cassiicola* and *S. lycopersici* were challenged with bacteria. Eleven isolated (E4, E6, E7, E8, E9, S15, S19, SE31, SE33, SE36, SE37 and Er-S) had an inhibitory effect on fungi which was evidenced by a reduction in colony diameter compared to the control (Figure 3). So, these eleven endphytes were selected to evaluate quantitatively their antagonist effect on the growth of fungal pathogens (Table 4).

Among the endophytes evaluated six provoked a major inhibition of fungal growth, they were E4 (*Micrococcus sp.*), E7 (*Bacillus sp.*), E8 (*Paenibacillus polymyxa*), E9 (*Bacillus sp.*), S15 (*Bacillus sp.*) and SE37 (*Bacillus sp.*) and were selected to follow antagonism assays (Table 4).

3.2.2. Activity of cell-free supernatant of endophytic bacteria against fungi pathogens.

We further evaluate the activity of cell-free supernatant from culture of endophytes E4, E7, E8, E9, S15 and SE37 against the growth of the fungal pathogens, *A. alternata*, *C. cassiicola* and *S. lycopersici*. The cell-free supernatants of isolates E7 and Er/S effectively inhibit fungal growth (Figure 4). This inhibitory effect against *C. cassiicola* was linked to the concentration of the cell-free

supernatants since only when the concentration was above 1 %, culture supernatants inhibited growth of *C. cassiicola* (Figure 4B).

The mycelial growth of *Alternaria*, *Stemphylium* and *Corynespora* were inhibited by cell-free supernatants of isolate E7 by 80, 75 and 27 %, respectively; while supernatants of Bacillus Er/S inhibited the mycelial growth by 70, 72 and 27 %, respectively (Figure 4 A, B, C). Interestedly, fungi exposed to culture supernatant presented morphological alterations such as wall thickness in the hypha and swollen mycelia (data not show).

3.2.3. Effect of volatiles from endophytic bacteria on fungal pathogen growth.

Isolates E7, E9, S15, SE37 and ER/S released antifungal volatile compounds (VOCs) that inhibited growth of *A. alternata*, *C. cassicola* and *S. lycopersici* at 72 - 144 h after inoculation (Figure 5).

Isolates E7, E9, and Er/S, all identified as representatives of *Bacillus sp.* inhibited growth of *A. alternata* by 68, 51 and 82 %, respectively (Figure 6A). They also inhibited growth of *C. cassicola* by 61, 42 and 82 %, respectively (Figure 6B); and of *S. lycopersici* by 48, 61 and 89 %, respectively (Figure 6C).

3.3. Plant material, growth conditions, inoculation with bacteria isolates and growth promotion assays.

In order to determine if endophytes of tomato seeds and seedlings have the potential to promote plant growth, the 21 isolates initially identified taxonomically were evaluated for their ability to promote growth of tomato plants, in plants cultured axenically in Crone tubes (glass, 3 cm diameter, 25 cm long). They were compared with growth of un-inoculated controls and plants inoculated with *Peudomonas fluorescens*. Seedlings length and dry weight were determined. From these results, a subset of strains was selected with which the test in pots was repeated.

Isolates E4 (*Micrococcus*), E6 (*Bacillus*), E8 (*Paenibacillus polymyxa*), S15 (*Bacillus*), S21 (*Brevundimonas*), SE28 (*Acinetobacter*), SE31 (*Microbacterium*), SE36 (*Psychrobacillus*), SS38 (*Bacillus*) y SS39 (*Bacillus*) were selected, based on the previously evaluated parameters. Among the selected strains it was considered in addition to the results of the previous screening, to employ at least two representatives of seed isolates and seedlings of each cultivar.

Subsequently, a new experiment was carried out in pots where we determined root volume, dry weight root as well as shoots. As a positive control a set of plants were inoculated with *P. fluorescens*.

Plants that had not pathogenic symptoms and that were inoculated with isolates E4, E6, E8, S15, SE31 and *P. fluorescens* had a higher root (RFW), root dry weight (RDW) and root volume (RV) as well as a higher shoot dry weight compared to non-inoculated plants (Figure 7). Plants inoculated with isolate E6, SE31 and *P. fluorescens* had no effect on plant growth RV and RDW respectively. While only two isolates, SS38 and SS39, promoted shoot growth, the rest of the isolates had no effect on plant growth (Figure 7).

Bacterial isolates also were evaluated for their ability to solubilize P, synthetize phytohormones and siderophores. Isolates E7, E8, S15, S19, S27, SE28, SE35, SE36, SE37 and SS38, proved to produce IAA. Regarding siderophores production, isolates E7 and SE28 proved to synthetize such compounds and only isolate E7 solubilized P (Table 5).

The isolates that had the highest potential to promote plant growth (E4, E6, E8, S15 and SE31) were evaluated in terms of biofilm formation (biofilm and autoaggregation), since such as characteristics might be indicative of a better colonization capacity.

Autoaggregation of bacteria behaved similarly to biofilm formation, heterogeneity was quite high; while some strains autoaggregation strongly others hardy did no (Table 6).

We conducted a correlation analysis to determine whether their ability of autoaggregation and biofilm formation of the strains was quantitatively related. A scatter plot was generated (Figure 8), and the Pearson correlation coefficient (r) was calculated. We observed an inverse correlation

between both phenotypes ($r \geq -0.64$, $P \leq 0.05$), maybe because cell-cell interactions of biofilm formation and aggregates not depend equally on the same physical adhesive forces.

3.4. *Figures, Tables and Schemes*

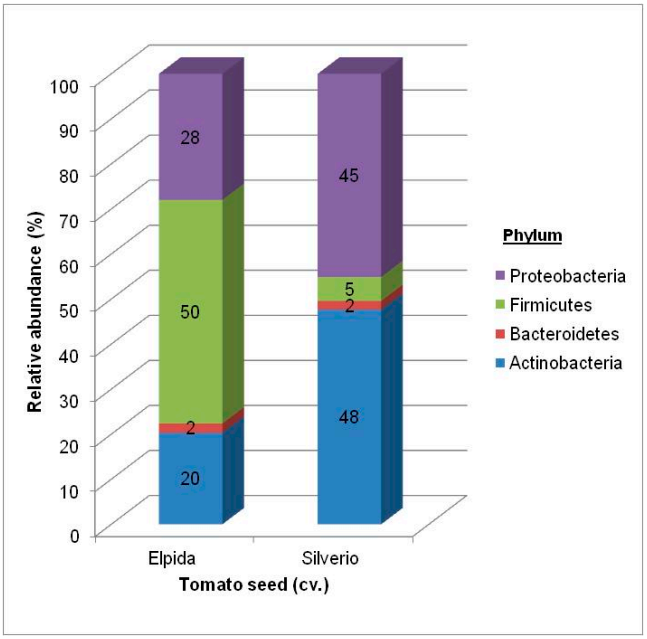


Figure 1. Taxonomic profiles of the bacterial community in each system at phylum level with the relative abundance (> 0.5 %).

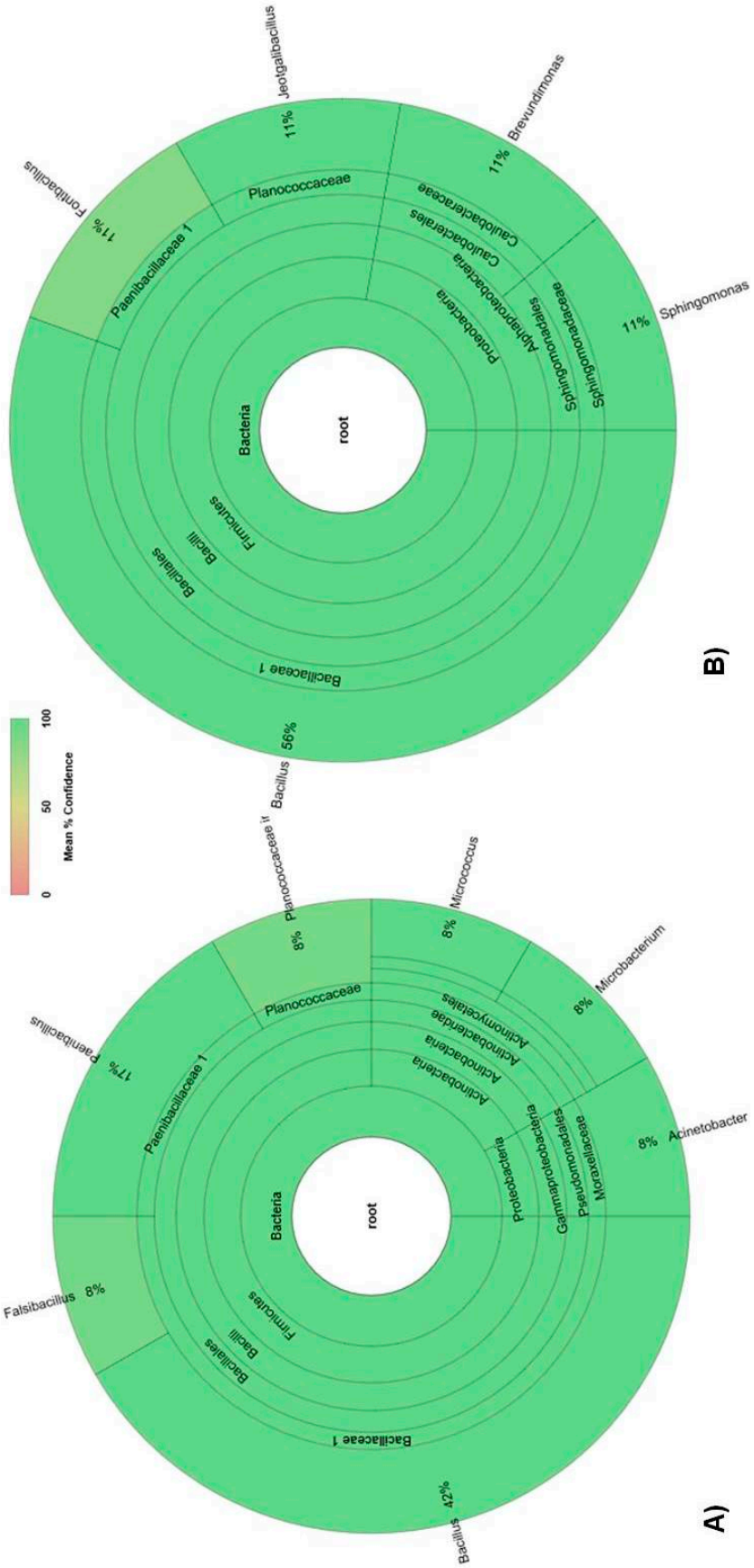
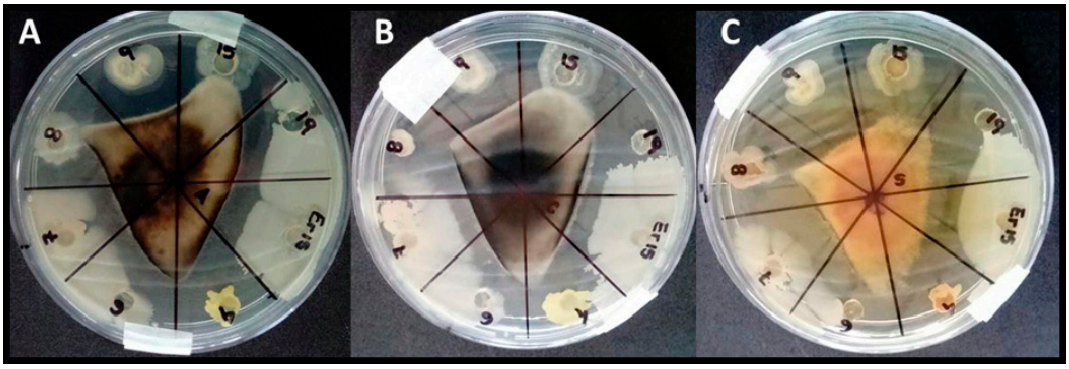


Figure 2. Comparison of bacterial species isolated from seeds and seedling of each tomato cultivar: A): Elpida cultivar, B): Silverio cultivar. 16S biodiversity graph generated with 16S Biodiversity tools of Genious software (version R9.0.5, Biomatters, http://www.geneious.com).



400

401 **Figure 3.** Antagonism effect of seven endophytes of seed (E4, E6, E7, E8, E9, S15, S19 and Er/S as
402 control), against three fungal pathogens of tomato *in vitro*: A) *Alternaria alternata*, B) *Corynespora*
403 *cassicola*, C) *Stemphylium lycopersici*.

Figure 4. Inhibitory activity of the three concentration of cell-free supernatant of endophytic bacteria (E4, E7, E9, S15, SE37 and Er/S as control) against fungi pathogens: A) *Alternaria alternata*, B) *Corynespora cassiicola*, C) *Stemphylium lycopersici*.

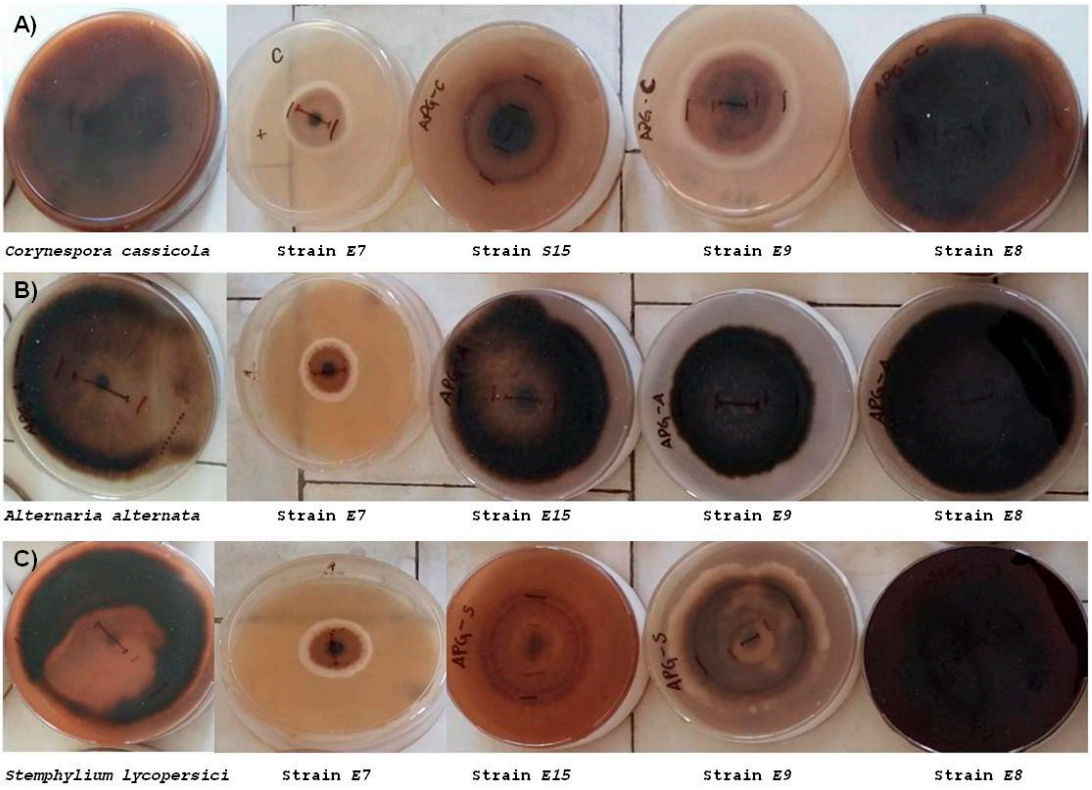
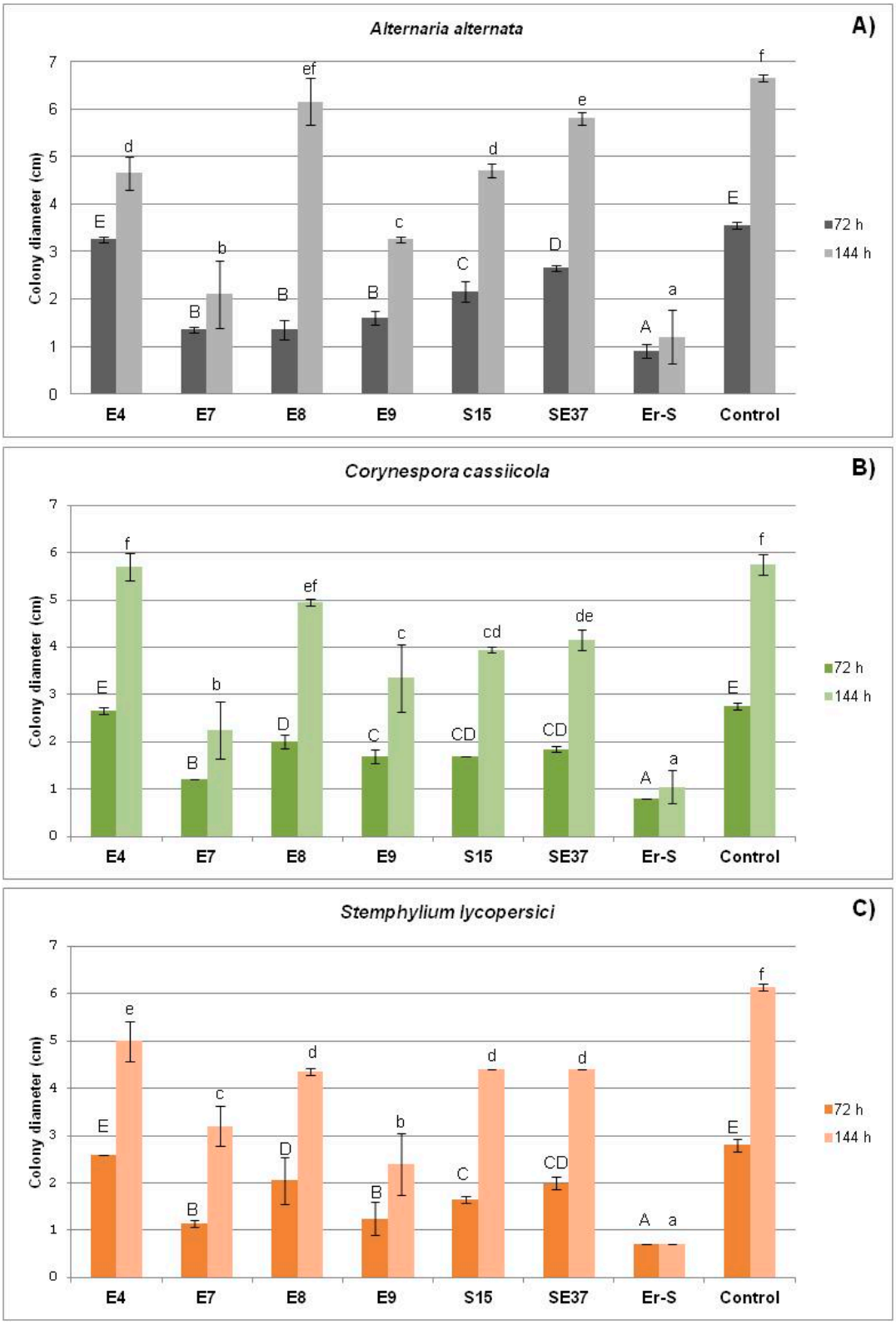


Figure 5. Effect on fungal pathogens growth of VOCs produce by entophytic bacteria, E7, S15, E9 and E8, against fungi pathogens: A) *Corynespora cassiicola*, B) *Alternaria alternata*, C) *Stemphylium lycopersici*.



412

413 **Figure 6.** Antifungal activities of volatiles from endophytic bacteria (E4, E7, E8, E9, S15, SE37 and
414 Er/S as control) against fungi pathogens: A) *Alternaria alternata*, B) *Corynespora cassiicola* and C)
415 *Stemphylium lycopersici*.

416

417

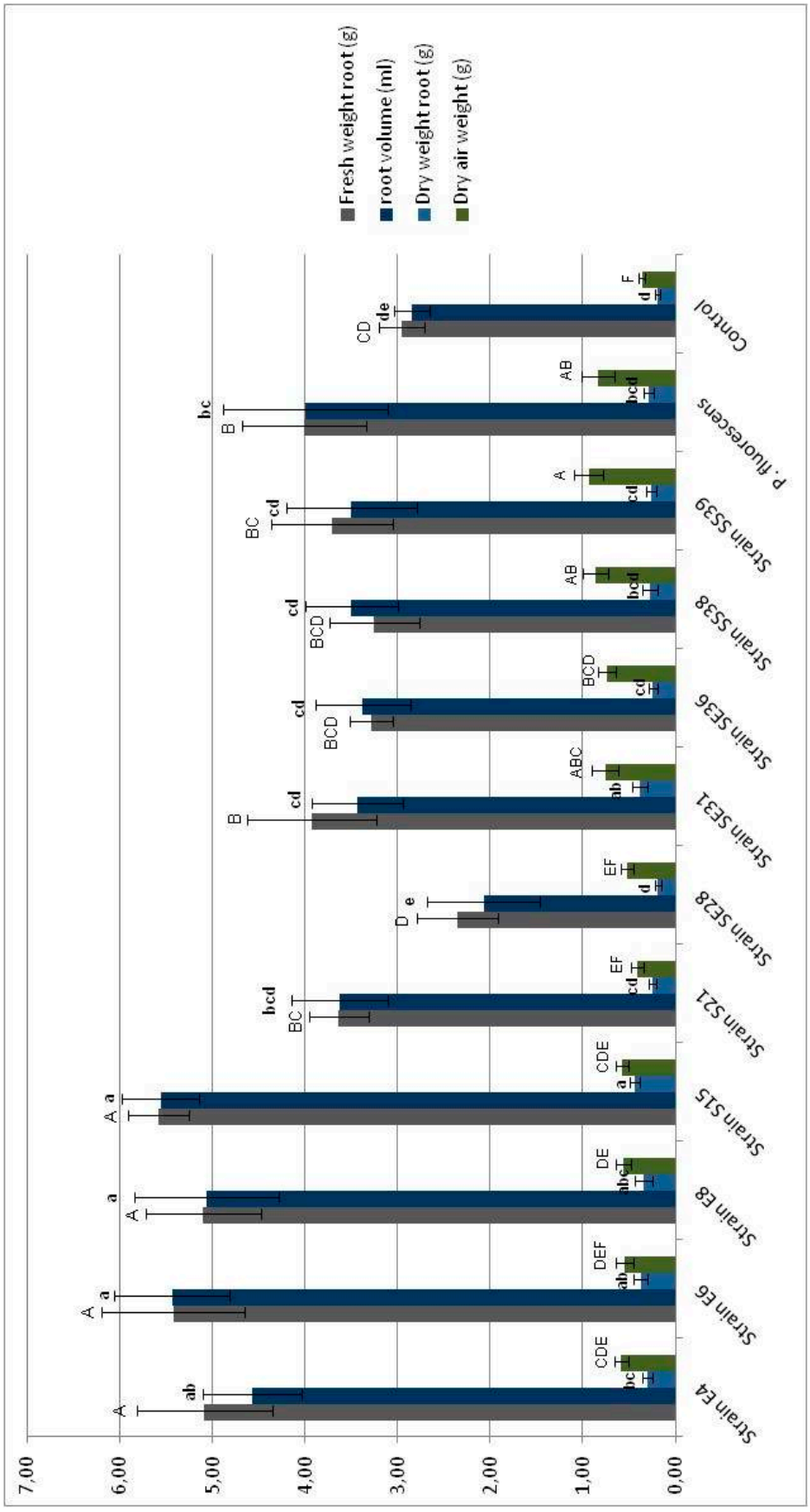


Figure 7. Effect on tomato plant growth (RDW, RFW, RV and ADW) produce by entophytic bacteria isolated from seed (E4, E6, E8, S15, S21) and seedling (SE28, SS38, SS39) of tomato.

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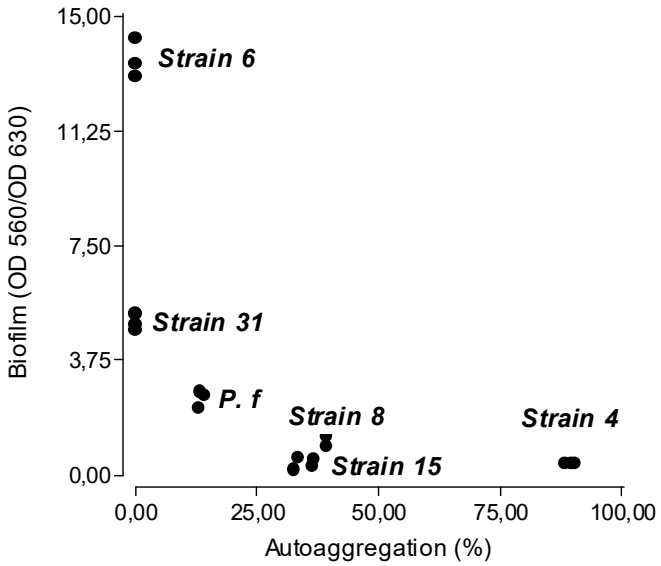

Pearson correlation coefficient (r)

Variable (1)	Variable (2)	n	Pearson	p-valor <0.05
Biofilm	Autoaggregation	18	-0.64	0.0046

Figure 8. Scatter plot of two variables: autoaggregation (percent) and relative biofilm formation ability (OD_{560nm}/OD_{630nm}).

Table 1. Average diversity estimates of the different communities studied.

Sample	Total sequences	Good's coverage (%)	^0H	^1H	^2H
Elpida seed	10,254 12,735	90	35,147	3.6	1.37
Silverio seed	11,496 12,838	90	62,867	3.7	1.39

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Table 2. The composition of the endophytic communities of Elpida and Silverio seed and seedling at order and genus level from illumine data set..

Phyla	Class	Order Elpida Seed	Genus Elpida Seed	Order Silverio Seed	Genus Silverio Seed
Actinobacteria	Actinobacteria	Actinomycetales 14.3 %	*Clavibacter* (61 %) *Corynebacterium* (20 %) *Micrococcus* (11 %) *Curtobacterium* (6 %) *Microbacterium* (2 %)	Actinomycetales 27.3 %	*Clavibacter* (81 %) *Corynebacterium* (6 %) *Micrococcus* (3 %) *Curtobacterium* (7 %) *Microbacterium* (3 %)
Bacteroidetes	Flavobacteria	Flavobacteriales 0.7 %	Flavobacterium (30 %)	Flavobacteriales 1.3 %	Flavobacterium (54 %)
	Sphingobacteria	Sphingobacteriales 0.5 %	Sphingobacterium (100 %)	Sphingobacteriales 1.7 %	Sphingobacterium (100%)
Firmicutes	Bacilli	Bacillales 63.3 % Lactobacillales 0.5 %	*Paenibacillus* (92 %) *Staphylococcus* (8 %)	Bacillales 2,7 % Lactobacillales 0,7 %	*Paenibacillus* (26 %) *Staphylococcus* (74 %)
Proteobacteria	Alpha	Rhizobiales 2,7 % Sphingomonadales 0,7 %	*Shinella* (70%) *Sphingobium* (15 %) *Rhizobium, Ensifer, Sinorhizobium* (15 %)	Rhizobiales 16.0 % Sphingomonadales 3.3 %	*Shinella* (70%) *Sphingobium* (15 %) *Rhizobium, Ensifer, Sinorhizobium* (15 %)
	Beta	Burkholderiales 0.5 %	*Achromobacter* (20 %) *Acidovorax* (80 %)	Burkholderiales 5.0 %	*Achromobacter* (29 %) *Acidovorax* (71 %)
	Gamma	Enterobacteriales 0.6 % Pseudomonadales 14.6 %	*Pantoea, Pectobacterium, Serratia* (3 %) *Pseudomonas* (75 %) *Moraxella* (14 %) *Acinetobacter* (8 %)	Enterobacteriales 4.0 % Pseudomonadales 37.3 %	*Pantoea, Pectobacterium, Serratia* (10 %) *Pseudomonas* (89 %) *Moraxella* (0.5 %) *Acinetobacter* (0.5 %)

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Table 3. Identification of bacterial isolates obtained from tomato seeds and seedling by the sequences of the *16S rDNA* gene sequence.

Isolate (origin)	Closest match in NCBI database (Accession number)	Identity (%)
E4 (seed Elpida)	*Micrococcus sp.* (MG963203)	99
E6 (seed Elpida)	*Bacillus sp.* (MG963204)	92
E7 (seed Elpida)	*Bacillus sp.* (MG963205)	99
E8 (seed Elpida)	*Paenibacillus polymyxa* (MG963206)	99
E9 (seed Elpida)	*Bacillus sp.* (MG963207)	98
S15 (seed Silverio)	*Bacillus sp.* (MG963209)	99
S19 (seed Silverio)	*Bacillus sp.* (MG963210)	99
S20 (seed Silverio)	*Sphingomonas sp.* (MG963211)	96
S21 (seed Silverio)	*Brevundimonas sp.* (MG963212)	99
S26 (seed Silverio)	*Paenibacillus sp.* (MG963213)	91
S27 (seed Silverio)	*Jeotgalibacillus sp.* (MG963214)	99
SE28 (seedling Elpida)	*Acinetobacter sp.* (MG963215)	98
SE31 (seedling Elpida)	*Microbacterium sp.* (MG963216)	99
SE33 (seedling Elpida)	*Paenibacillus sp.* (MG963217)	99
SE34 (seedling Elpida)	*Bacillus sp.* (MG963218)	99
SE35 (seedling Elpida)	*Bacillus sp.* (MG963219)	99
SE36 (seedling Elpida)	*Psychrobacillus sp.* (MG963220)	97
SE37 (seedling Elpida)	*Bacillus sp.* (MG963221)	98
SS38 (seedling Silverio)	*Bacillus sp.* (MG963222)	99
SS39 (seedling Silverio)	*Bacillus sp.* (MG963223)	99
SS41 (seedling Silverio)	*Bacillus sp.* (MG963224)	96
Er/S	*Bacillus subtilis* (MG963208)	99

Table 4. Determination quantitative of antagonist effect in the growth of fungi.

Strain	*Alternaria alternata*	*Corynespora cassiicola*	*Stemphylium lycopersici*
SE37	1,65 ± 0,289 ^{a}	2,95 ± 0,06 ^{bc}	1,4 ± 0,231 ^{a}
E4	2,05 ± 0,289 ^{ab}	2,55 ± 0,289 ^{a}	1,4 ± 0,231 ^{a}
E8	2 ± 0,115 ^{ab}	2,8 ± 0,115 ^{ab}	1,55 ± 0,06 ^{ab}
E7	2,35 ± 0,173 ^{bc}	2,85 ± 0,06 ^{ab}	1,85 ± 0,06 ^{bc}
Er/S	2,45 ± 0,404 ^{bc}	3 ± 0,08 ^{bc}	1,9 ± 0,115 ^{bc}
S15	2,55 ± 0,173 ^{bc}	3,25 ± 0,289 ^{c}	2 ± 0,115 ^{cd}
E9	2,75 ± 0,289 ^{cd}	2,7 ± 0,115 ^{ab}	2,25 ± 0,06 ^{cde}
E6	2,9 ± 0,115 ^{cde}	3,6 ± 0,115 ^{d}	2,35 ± 0,404 ^{def}
S19	2,9 ± 0,08 ^{cde}	3,7 ± 0,115 ^{d}	2,6 ± 0,115 ^{efg}
SE31	3,15 ± 0,289 ^{def}	3,7 ± 0,115 ^{d}	2,6 ± 0,115 ^{efg}
Control	3,6 ± 0,08 ^{f}	4,05 ± 0,06 ^{e}	2,75 ± 0,06 ^{fg}
SE33	3,35 ± 0,06 ^{ef}	4,05 ± 0,06 ^{e}	2,9 ± 0,115 ^{g}
SE36	3,25 ± 0,289 ^{def}	4,3 ± 0,08 ^{e}	2,95 ± 0,06 ^{g}

433 **Table 5.** IAA and Siderophore production and Phosphate solubilizacion.

Isolate[1]	Source	IAA production	Siderophore production	Phosphate solubilization
E7	Seeds Elpida	+	+	+
E8		+		
S15		+		
S19	Seeds Silverio	+		
S27		+		
SE28		+	+	
SE35	Seedling Elpida	+		
SE36		+		
SE37		+		
SS38	Seedling Silverio	+		

434 [1] Isolates E4, E6, E9, S20, S21, S26, SE31, SE33, SE34, SS39 and SS41 are not presented in the table
435 because they had a negative phenotype for these characteristics evaluated.

436 **Table 6.** Biolfim and autoaggregation formation ability of entophytic bacteria with potential to plant
437 growth promotion..

Isolate	Biofilm (OD_{560nm}/OD_{630nm})	Autoaggregation (%)
E4	0.38 + 0,02	89.41 + 1.08
E6	13.58 + 0.62	0
E8	0.44 + 0.23	34.16 + 2.33
S15	0.86 + 0.52	38.14 + 1.55
SE31	5.00 + 0.26	0
PF	2.51 + 0.26	13.54 + 0.55

438 **4. Discussion**

439 Endophytic bacteria are microorganisms that can colonize plants tissues intercellularly and
440 healthfully coexist with in plant tissues [45]. Seeds are the main structure of plants for the species
441 survival along time and play a key role in agriculture [46]. They are the vehicle of a variety of
442 pathogens and beneficial bacteria [15]. When seeds germinate, growth of these endophytic microbial
443 communities occurred [47, 48], and they might be enriched with microorganisms originated in soils.
444 We analyzed the communities of bacterial endophytes in seeds of two cultivars of Tomato by
445 metagenomic analysis and by isolating culturable endophytes. Seeds of tomato had a rather low
446 number of species which additionally was found to be in both cultivars of tomato in a somewhat
447 similar way of other plant species [49-52]. However, Elpida and Silverio host significantly different
448 endophytic communities regarding the composition to the order level, these might be due to the
449 different genotypes of the cultivars investigated. Simon et al. [53] found that growth of both intrinsic
450 and inoculated bacteria were different in tomato genotypes. So, even though seeds posses similar
451 endophytic communities, our results confirmed that the plant genotype has an impact on the
452 structure of the endophytic bacterial community, which makes sense considering that each genotype
453 might secrete a wide array of different nutrients and molecules to the apoplastic environment.
454 Adams and Kloepper [54] investigated the impact of cotton plant genotype on the endophytic
455 population of seeds, stems and roots. They found that cotton plants have endophytic bacterial
456 communities that change throughout the process of germination and seedling development, and
457 cotton cultivars harbor different endophytic bacterial community structures.
458 Culturable bacteria isolated from seeds and seedlings of both cultivars of tomato were similar
459 regarding the phyla detected inside seeds and seedlings. These suggest that tomato seeds might
460 contain a basic subset of bacteria that entre into the seeds along the reproductive development and

that might play specific roles whether this relates with seed health or seedling growth promotion. Seeds were mostly colonized by Firmicutes, this phylum also increased within seedling suggesting that seed germination provide somehow a nutritional advantage. Among the species found were *Bacillus*, *Paenibacillus*, *Psychrobacillus* y *Jeotgalibacillus* are capable of forming endospores, which might explain their high representation in seed. So, the ability to form endospores confers an advantage of seed colonizers as this assures their survival in storage seeds [50, 21]. We have not analyzed the bacterial population of physiologically matured seeds immediately after development that might contain more diverse bacteria that may die along seed stored with low water content. Probably the seed maturation process do not select bacteria based on their properties, but the diversity, at least of the culturable ones, seems to be influenced by their ability to sporulate. Mano et al. [49] found Gram negative isolates predominated in the early stages of seed development and Gram positive isolates appeared as seeds mature. In this regard, we isolated 18 % of Gram negative (*Sphingomonas* and *Brevundimonas*) and 82 % of Gram positive bacteria on seeds (Elpida and Silverio), while developed seedlings (Elpida and Silverio) 90 % of bacteria were Gram positive species and only 10 % Gram negative (*Acinetobacter*). Evidently, some changes occur along seedling development that promotes certain groups of microorganisms [21]. Seed development might have a high requirement of nutrient generating in this way a stressful environment for bacteria and, as result of this only those able to sporulate survive this stringent environment much better. We also evaluated the capacity of endophytics bacteria of seeds to colonizer or move along the plant. Bacterial endophytes of tomato carried by migration from the endosperm to the radicle.

Common bacterial genera reported in seeds are *Bacillus* and *Pseudomonas*. Also *Paenibacillus*, *Micrococcus*, *Staphylococcus*, *Pantoea* and *Acinetobacter* are often found inhabiting seeds [18, 49, 50, 51, 52, 19, 21]. Tomato seeds host endophytic bacterial communities similar to these reported for others plant species, suggesting that their presence is either essential is due to their strategy of dispersion within environment.

Endophytes most probably provide benefits host plants through various mechanisms, such as the synthesis of antimicrobial substances or the synthesis of plant growth promoters. Our result showed that not all bacteria (E4, E7, E8, E9, S15 and SE37) inhibited mycelial growth, under controlled conditions; however, they differ in their ability to synthesize some inhibitory molecules. *Bacillus sp.*, E7 inhibited growth of three soil-borne plant pathogens (*A. alternata*, *C. cassicola* and *S. lycopersici*) and it did so by means of water soluble inhibitory products that are released to the culture medium; and also by the synthesis of VOCs products. So, this bacteria has different strategy through we cannot assess which is the most important one in nature.

Antifungal molecules synthesized by microorganism may be used to biocontrol microorganism [38]. Most biocontrol products synthesized by some species of *Bacillus* are small polypeptides, as iturins and bacillomycins. Theses antifungal peptides inhibit the growth of the large number of fungi [38].

In this work, we found that *Bacillus* species colonize tissues of tomato seedlings, in this regard one isolates E7 presented outstanding capacity to protect plants against fungal pathogens.

Another potential role of microorganisms was characteristic of isolates E4 (*Micrococcus*), E6 (*Bacillus*), E8 (*Paenibacillus polymyxa*), S15 (*Bacillus*) and SE31 (*Microbacterium*), as they all promoted of plant growth. Representatives of these genera have already been found within plant tissues also promoting plant growth. Actinobacteria like *Micrococcus* and *Microbacterium*, are frequently found within the rhizosphere of plant, suggesting that they play crucial roles while interacting with plant that leads to plant growth promotion [55]. Sangthong et al. [56] found that representatives of *Micrococcus sp*. promoted root and shoot length, as well as shoot biomass of *Zea mays* L. The isolate proved to be a potent bioaugmenting agent, facilitating cadmium phytoextraction in *Z. mays* L. Prapagdee et al. [57] found that *Micrococcus sp.* promote growth and cadmium uptake in cadmium polluted soil by dicotyledonous plant. In this work, we also found that *Micrococcus* and *Microbacterium*, E4 and SE31 respectively, promoted root and shoot growth of tomato plants. Vílchez et al. [58] showed that in pepper plants *Microbacterium sp*. promoted an increase in sugar biosynthesis that provided plants with a more efficient osmotic adjustment relieving in this way

plants from the effect of stress on the host plants. Also *Microbacterium sp*. protects plants against drought stress while living within plants [58]. The plant growth promotion and protection effect of *Bacillus* and *Paenibacillus* is the result of several complex and interrelated processes that involve direct and indirect mechanisms such as nitrogen fixation, phosphate solubilization, siderophore production, phytohormone production and control of plant diseases [59-61]. Also in this work bacteria play several different roles. *Bacillus* isolates E6 and S15 and *P. polymyxa* isolates E8 are plant growth promoters. Furthermore, S15 and E8 also have a capacity to control fungi pathogens of Tomato. Such bacteria share groups of key features like high secretion capacity, spore formation capacity being the latter features critical for the commercial applications with a long shelf life [50, 21, 61].

One of the key steps while using bacteria as biocontrollers is the effective colonization of plant roots, particularly to promote growth. Bacteria persist in natural environments by forming biofilms. Biofilms are highly structured, surface-attached communities of cells encased in a self-produced extracellular matrix [62, 43]. We found these 5 isolates highly efficient to promote plant growth that form some type of biofilm, which might provide an adaptive advantage colonize plant tissues. Still in some cases like *P. polymyxa*, biofilm development in the root tips was crucial for bacteria to penetrate intercellular spaces; however bacteria did not spread within plant tissues suggesting that other crucial mechanisms are needed [62].

5. Conclusions

The community associated with seeds of different cultivars reflects the different resources and its potential to prevent the attack of pathogens and promote plant growth. The use of tools like metagenomics allowed us to know more about the community associated to the different cultivars, turning out to be a useful technology. Different cultivars of tomato (genotypes) host significantly different endophytic communities regarding the composition to the order level these might be due to their different genotypes.

We conclude that the ndophytic bacteria isolated from cultivars Elpida and Silverio are the source of organisms that synthetize antifungal substances that could potentially be used in the biocontrol of fungi that commonly produce diseases in the tomato crop.

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Author Contributions:

"Silvina López and Graciela Pastorino conceived and designed the experiments; Silvina López, Graciela Pastorino, Gustavo Lucentini and Mario Franco performed the experiments; Silvina López and Rocio Medina analyzed the metagenomic data; Mario Saparrat and Pedro Balatti contributed reagents/materials/analysis tools; Silvina López and Pedro Balatti wrote the paper."

Conflicts of Interest:

"The authors declare no conflict of interest."

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