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Original article

Novel components of leaf bacterial communities of field-grown tomato plants and their potential for plant growth promotion and biocontrol of tomato diseases

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Abstract

This work aimed to characterize potentially endophytic culturable bacteria from leaves of cultivated tomato and analyze their potential for growth promotion and biocontrol of diseases caused by *Botrytis cinerea* and *Pseudomonas syringae*. Bacteria were obtained from inner tissues of surface-disinfected tomato leaves of field-grown plants. Analysis of 16S rRNA gene sequences identified bacterial isolates related to *Exiguobacterium aurantiacum* (isolates BT3 and MT8), *Exiguobacterium* spp. (isolate GT4), *Staphylococcus xylosus* (isolate BT5), *Pantoea eucalypti* (isolate NT6), *Bacillus methylotrophicus* (isolate MT3), *Pseudomonas veronii* (isolates BT4 and NT2), *Pseudomonas rhodesiae* (isolate BT2) and *Pseudomonas cichorii* (isolate NT3). After seed inoculation, BT2, BT4, MT3, MT8, NT2 and NT6 were re-isolated from leaf extracts. NT2, BT2, MT3 and NT6 inhibited growth of *Botrytis cinerea* and *Pseudomonas syringae pv. tomato* in vitro, produced antimicrobial compounds and reduced leaf damage caused by *B. cinerea*. Some of these isolates also promoted growth of tomato plants, produced siderophores, the auxin indole-3-acetic and solubilized inorganic phosphate. Thus, bacterial communities of leaves from field-grown tomato plants were found to harbor potentially endophytic culturable beneficial bacteria capable of antagonizing pathogenic microorganisms and promoting plant growth, which could be used as biological control agents and biofertilizers/biostimulators for promotion of tomato plant growth.

Keywords: Endophytic culturable bacteria; *Botrytis cinerea*; *Pseudomonas syringae pv.tomato*; Biocontrol; Growth promotion; Antimicrobial compounds

1. Introduction

A wide diversity of plants is associated with endophytic bacteria that colonize host tissues internally without causing damage or eliciting disease symptoms [1]. Although the mechanisms that govern the interaction between plants and bacterial endophytes are less well known than those involved in other plant-microbe interactions, there is ample evidence that many endophytic bacteria exert beneficial effects on plants [2,3]. In this way, some bacterial endophytes act as plant growth-promoting bacteria (PGPB), which stimulate plant growth by several mechanisms. Mechanisms of growth promotion by well-studied PGPB include phosphate solubilization, production of phytohormones, nitrogen fixation (performed by diazotrophic microorganisms) and production of siderophores that contribute to the transportation of ferric iron into plant cells [4,5]. PGPB can also stimulate plant growth by preventing the deleterious effects of phytopathogenic microorganisms, acting as biological control agents, which can exert their activity by direct antagonistic effects on pathogenic organisms or indirectly, by eliciting plant defense responses. In this way, competition for a substrate or an ecological niche, production of inhibitory compounds and induction of systemic

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resistance on the plant host can mediate biological control of plant diseases [4,6,7]. The above-mentioned features of PGPB raise interest in their agronomic potential, in the light of public concern about the use of agrochemicals and the need to find alternative methods for increasing plant yield and protection against pathogenic microorganisms. The best-known PGPB are plant growth-promoting rhizobacteria (PGPR) that colonize the rhizosphere and, in some cases, also enter the root interior and establish endophytic populations [8]. Some PGPR are able to pass the root endodermis barrier and reach the vascular system, subsequently colonizing other plant organs as endophytes [9]. Even though endophytic bacteria usually enter plants through roots, endophytic communities can also originate from the phyllosphere, the anthosphere and the spermosphere [2]. Regardless of the way in which bacterial endophytes get into the plant, current knowledge about the mechanisms that regulate colonization of aerial organs by these microorganisms is scant, as compared to pathogenic bacteria. Moreover, the diversity and potential of leaf endophytic bacteria for growth promotion and biological control of plant diseases in the phylloplane is far from being well known.

The cultivated tomato (*Solanum lycopersicum* L., formerly *Lycopersicon esculentum* Miller), a fruit considered to be a vegetable, is widely grown and constitutes a major agricultural industry worldwide. In addition, this species is well studied in terms of genetics, genomics and breeding, thus being an excellent model system for basic and applied research. Diseases are one of the main problems in the tomato industry throughout the world and provoke significant economic losses (http://faostat.fao.org). In this regard, the susceptibility of tomato to many phytopathogenic microorganisms has led to intense use of agrochemicals. Among other pathogens, the cultivated tomato is attacked by *Botrytis cinerea* Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel). This phytopathogenic fungus is the causal agent of gray mold, which also affects more than 200 crop species worldwide. Tomato plants can become infected either after penetration of stems and leaflets through scars or wounded tissue, as well as after colonization of senescent tissues [10]. As a consequence, all above-ground parts of the plant can be affected [11]. Phytopathogenic bacteria also cause significant losses in tomato production. In this regard, bacterial speck caused by *Pseudomonas syringae* pv. *tomato* is one of the most economically important bacterial diseases in many tomato-growing regions of the world [12]. Lesions occur on leaflets and may spread to stems, and flowers. Yield reductions can result from the reduced photosynthetic capacity of infected foliage, defoliation, flower abortion and fruit damage [13]. *B. cinerea* and *P. syringae* are also used as models for the study of pathogenicity mechanisms. Thus, the interest in these phytopathogenic microorganisms is not only based on the importance of the diseases caused by them, but also on their utility in understanding fundamental aspects of plant-pathogen interactions.

The composition and diversity of bacterial communities that endophytically colonize tomato leaves have previously been explored through 16S rRNA gene pyrosequencing [14], but information on the beneficial effects of leaf endophytic bacteria of tomato is still lacking. On this basis, and also considering the importance of cultivated tomato both as a crop and a model plant, the present work aimed to characterize potentially endophytic culturable bacteria from tomato leaves, and to analyze their potential for growth promotion and the control of leaf diseases caused by *B. cinerea* and *P. syringae*.

2. Materials and methods

2.1. Strains of pathogenic microorganisms, growth conditions and inoculation

B. cinerea strain B05.10 was stored as a conidial suspension at −80 °C in potato-dextrose broth (PDB) with 30% (v/v) glycerol. *P. syringae* pv. *tomato* DC3000 was kindly supplied by Dr Maria E. Álvarez (Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina) and was maintained at −80 °C in Luria-Bertani medium (LB) supplemented with 50 mg l^{-1} rifampicin and 20% (v/v) glycerol.

For plant inoculation, *P. syringae* was grown in liquid LB containing rifampicin (50 mg l^{-1}) at 28 °C. Bacterial cells were centrifuged and resuspended in sterile 0.01 mol l^{-1} $MgCl_2$ pH 7.0. Bacterial density was adjusted to 10^8 CFU ml^{-1} (OD_{600} = 0.1) and bacterial suspensions thus obtained were sprayed on the leaf surface. Leaves sprayed with sterile 0.01 mol l^{-1} $MgCl_2$ pH 7.0, were used as controls.

For plant inoculation with *B. cinerea*, conidia were collected from 10- to 15-day-old cultures with sterile water containing 0.02% (v/v) Tween 20, the suspension was then filtered and the conidia concentration was adjusted to 5×10^4 conidia ml^{-1} with PDB supplemented with 0.01 mol l^{-1} sucrose and 0.01 mol l^{-1} KH_2PO_4. The suspension was incubated for 3 h at room temperature. Two aliquots (5-μl each) of this suspension were inoculated on each side of the central vein of tomato leaves. The tomato cultivar 'Rio Grande' was used for the abovementioned experiments.

2.2. Isolation of bacteria from inner tissues of tomato leaves

A collection of bacteria associated with tomato leaves was obtained from plants of different cultivars grown in horticulture farms of the La Plata district in Buenos Aires Province, Argentina. Samples were obtained during springtime (November–December), 2009. A total of sixteen samples of tomato leaves were collected from four different farms. GPS coordinates for these farms were: farm 1, 34° 54′ 49″S, 58° 01′ 29″W; farm 2, 34° 54′ 57″S, 58° 02′ 24″W; farm 3, 34° 59′ 46″S, 58° 01′ 23″W; farm 4, 34° 56′ 44″S, 58° 08′ 00″W. Within each farm, four randomly distributed sampling sites were chosen and a single sample was taken from each sampling site. Each sample consisted of at least six leaves from different plants. The epiphytic microflora was eliminated by surface disinfection with 1.3% $HClO_4$ and 0.01% (w/v) Tween 20 for 10 min and rinsing (3×) with sterile distilled water. No bacterial or fungal growth was detected after plating aliquots of the final wash on tryptic

soy agar (TSA) (tryptone, 17.0 g l^{-1}; soya peptone, 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}), thus confirming the efficacy of disinfection in eliminating cultivable epiphytic microorganisms. Samples were homogenized in 0.01 mol l^{-1} $MgCl_2$ using an Omnimixer 17106® (OCI Instruments) and 100 µl aliquots were plated on TSA plates and incubated at 28 °C for 48 h. Colonies considered to be morphologically different on the basis of size, shape, and color were selected and subcultured to obtain pure cultures which were afterwards kept at −80 °C as glycerol stocks. A total of 65 isolates were obtained, part of which were analyzed. Thus, ten isolates (BT2, BT3, BT4, BT5, GT4, MT3, MT8, NT2, NT3 and NT6) found not to be redundant according to their 16S rRNA gene sequences and BOX-PCR profiles (next section) were selected for testing their ability to promote growth and their antagonistic activity towards *B. cinerea* and *P. syringae*. In order to confirm the ability of bacterial isolates to endophytically colonize tomato plants, seeds were surface-disinfected as indicated above and then inoculated with bacterial suspensions ($OD_{600} = 0.1$ in 0.01 mol l^{-1} $MgCl_2$ pH 7.0). Inoculated seeds were sown on MS and plants were grown under axenic conditions for three weeks. Leaf and root sections were then obtained and surface-disinfected in order to eliminate bacteria that could have grown on the surface of these organs. Leaf tissues were ground as described previously and 100 µl aliquots were plated on TSA. Root sections were also placed on TSA plates. Bacterial growth was analyzed after 48 h incubation at 28 °C.

2.3. Isolation of genomic DNA, PCR amplification and sequencing of 16S rRNA gene

Total DNA was extracted from bacterial isolates and nearly full-length (approximately 1,500 bp) 16S rRNA genes were amplified from isolates using primers 41f and 1488r [15]. PCR products were purified and sequenced using an Applied Biosystems ABI 377 sequencer. The 16SrRNA gene sequences determined in this study have been deposited in the GenBank database under accession numbers KT036452-KT036461.

Non-redundant isolates were identified by BOX-PCR fingerprinting [16] using the universal BOXA1R primer (Table S1). PCR amplification and electrophoretic analysis were carried out as described in Castagno et al. [17].

2.4. Phylogenetic analysis

Sequence analysis was performed with ClustalW software from the EMBL server. Aligned sequences were analyzed with MEGA 4.0 Software [18]. Phylogenetic analyses of the 16S rRNA sequences were performed by the UPGMA method [19]. The phylogenetic distances were computed by the p-distance method [20] and statistical support for tree nodes was evaluated by bootstrap analysis [21].

2.5. Siderophore production and phosphate solubilization

Siderophore production was determined on chrome-azurol S (CAS) medium following the Universal Chemical Assay [22]. To prepare 1 L of blue agar, 60.5 mg CAS were dissolved in 50 ml water and mixed with 10 ml iron(III) solution (0.001 mol l^{-1} $FeCl_3.6H_20$, 0.01 mol l^{-1} HCl). Under stirring, this solution was slowly added to 72.9 mg CTAB dissolved in 40 ml water and the resultant dark blue solution was autoclaved. A mixture of 780 ml H_20, 100 ml 10X MM9 salts (3 g l^{-1} KH_2PO_4, 5 g l^{-1} NaCl, 10 g l^{-1} NH_4Cl), 15 g agar, 30.24 g Pipes, pH was adjusted to 6.8 with NaOH and autoclaved. After cooling to 50 °C, 10 ml glucose (20% w/v), 1 ml $MgSO_4$ (1 mol l^{-1}), 1 ml $CaCl_2$ (100 mol l^{-1}), 4 ml thiamine (500 µg ml^{-1}), 4 ml nicotinic acid (500 µg ml^{-1}) and 100 ml of the abovementioned dark-blue solution were added. Bacterial isolates (overnight cultures) were spotted on the CAS medium thus obtained. After 48 h incubation at 28 °C, siderophore production was evidenced by the formation of orange to yellow haloes around the colonies. For Gram-positive bacteria, a modified protocol described by Milagres et al. [23] was used. Phosphate solubilization was determined as described by Castagno et al. [17]. Bacterial strains (16-h-old cultures) were spotted on plates containing National Botanical Research Institute phosphate growth medium (NBRIP) (5 g l^{-1} $MgCl_2.6H_2O$, 0.25 g l^{-1} $MgSO_4.7H_2O$, 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.

2.6. Phytohormone production

Bacterial cultures in stationary growth phase in AB medium (3 g l^{-1} K_2HPO_4; 1 g l^{-1} NaH_2PO_4; 1 g l^{-1} NH_4Cl; 0,3 g l^{-1} $MgSO_4$; 0,15 g l^{-1} KCl; 0,012 g l^{-1} $CaCl_2$; 0,01 g l^{-1} $FeCl_3$; 2,5 g l^{-1} glucose) were centrifuged and supernatants thus obtained were used for phytohormone analysis as described in detail by Perrig et al. [24]. Briefly, Zeatin (Z) was identified and quantified by reversed phase high performance liquid chromatography (HPLC) with UV detection (254 nm). For indole-3-acetic acid (IAA), abscisic acid (ABA) and gibberellic acid (GA_3) determinations, after an initial HPLC purification, these compounds were further identified and quantified by gas chromatography-mass spectrometry with selective ion monitoring. IAA production was tested both with and without the addition of 0.0075 mol l^{-1} tryptophan to the culture medium.

2.7. In vitro antagonism of bacterial isolates towards tomato pathogens

In order to test antagonistic effects towards *B. cinerea*, eight droplets (1 µl each) of overnight cultures of each bacterial strain were spotted at the periphery of PDA plates inoculated in the center with a 25-mm^2 plug of *B. cinerea* mycelium. Plates inoculated only with *B. cinerea* were used as controls and photographs were taken when mycelium reached the edge of control plates. The radius of *B. cinerea* colonies was measured using Image-ProPlus V 4.1 software (Media Cybernetics). Inhibition of *B. cinerea*

growth was calculated as % inhibition = [1- (diameter of colony confronted with bacterium/diameter of control colony] x 100.

Inhibition of *P. syringae* pv. *tomato* growth was measured by a dual inoculation technique described by Zhou et al. [25]. *P. syringae* cells obtained from liquid cultures were mixed with melted LB agar at 42 °C to a final concentration of 10^6 CFU ml^{-1}. This mixture was dispensed in Petri dishes and once the mixture solidified, a 5-µl aliquot of each endophytic bacterium (10^7 CFU ml^{-1}) was spot-inoculated into the center of the plate. Antagonistic activities were evaluated by measuring the widths of the inhibition zones of *P. syringae* around the colonies of antagonistic bacteria after incubating for 2 days at 28 °C.

2.8. Production of antimicrobial compounds by bacterial isolates obtained from tomato leaves

Cell-free culture filtrates were obtained after growing bacterial isolates in TSB up to stationary phase, centrifuging and filtrating through 0.2 µm membrane filters. Antifungal activity was analyzed on the basis of inhibition of germination and germ tube growth of *B. cinerea* conidia, as follows. Twenty-µl aliquots of supernatants were mixed with 20 µl of a conidia suspension (1.5×10^4 conidia ml^{-1}) in 0.025 mol l^{-1} potassium phosphate buffer pH 5 with 100 g l^{-1} glucose in multiwell plates, and were incubated at 22 °C. After 12–16 h, germination was stopped by adding 5 µl of formaldehyde and germinated conidia were counted under a binocular microscope. Conidia were considered as germinated if the length of the germ-tube was at least twice the diameter of the conidium. More than 100 conidia were counted per well and a completely randomized design with three replicate wells was used.

Production of antibacterial compounds by bacterial isolates was assessed by the ability of culture supernatants to inhibit growth of *P. syringae*. Three-milliliter cultures of *P. syringae* were supplemented with filtrates from each bacterial antagonist to a final filtrate concentration of either 10 or 50% (v/v). Growth of *P. syringae* was estimated by optical density measures (600 nm) during incubation at 28 °C.

2.9. Plant material, growth conditions, inoculation with bacterial isolates and growth promotion assays

Tomato plants (cultivar *Río Grande*) were routinely cultured as follows. Seeds were disinfected with 70% (v/v) ethanol for 20 min, 1.3% sodium hypochlorite for 30 min and then were rinsed (×5) with sterile distilled water. Seeds were dispensed in pots (1 seed/pot) filled with a mixture of sand and perlite (1:1), and were irrigated with sterile ¼ Hoagland solution [26]. Plants were grown for 5–6 weeks in a growth chamber with a 16/8 h photoperiod at 24/21 ± 2 °C and 55/75 ± 5% relative humidity (day/night) and a photon flux density of 200 µmol m^{-2} s^{-1}.

For experiments that involved seed inoculation with bacterial isolates, disinfected seeds were immersed in a bacterial suspension for 30 min at room temperature with periodic shaking. Plants derived from these seeds were re-inoculated by dispensing 5 ml of the bacterial suspension at the stem base 15 days after sowing. Bacterial suspensions were obtained from overnight cultures that were centrifuged and resuspended in sterile 0.01 mol l^{-1} $MgCl_2$ pH 7.0 to an OD of 0.1. Plants derived from seeds immersed in 0.01 mol l^{-1} $MgCl_2$ were used as controls.

Growth promotion by bacterial isolates was analyzed in plants inoculated and cultured as described above. Fresh weight of stems and roots was determined after a 6-week growth period.

2.10. Biocontrol assays on detached leaves and whole plants

Detached leaflets from 6 week-old plants, which were dispensed on Petri dishes containing 0.8% (w/v) water-agar, were used. For *P. syringae pv. tomato* DC3000 bioassays, leaflets were syringe-infiltrated on the abaxial side with 600 µl of a suspension of the antagonistic bacterium (OD = 0.1) and afterwards were inoculated with *P. syringae* pv. *tomato* DC3000. Two, three and four days after inoculation, 100 mg samples of tissue were taken from the middle of the leaflets and were ground into 0.01 mol l^{-1} $MgCl_2$. Dilutions of the extracts thus obtained were plated on LB agar supplemented with 50 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin. The titers of *P. syringae* pv. *tomato* DC 3000, which is resistant to both of these antibiotics, were determined as the number of CFU detected after incubation at 28 °C for 48 h. Previously, antagonistic bacteria were confirmed not to be resistant to rifampicin. Five replicate leaflets were analyzed and leaflets inoculated only with *P. syringae* were used as controls in multiplication of this bacterium in the absence of other microorganisms.

For *B. cinerea* bioassays, leaflets were infiltrated with endophytic bacteria as described in previous paragraphs and inoculated with *B. cinerea*. The mean size of the necrotic area around the inoculation site was determined using Image-ProPlus V 4.1 software (Media Cybernetics) on ten replicates. Leaflets inoculated only with *B. cinerea* were used as controls of *B. cinerea* infection.

An alternative approach for testing the ability of bacterial isolates to antagonize the above-mentioned pathogens consisted of their inoculation on seeds at sowing. Leaflets were detached from 6-week-old plants grown from seeds inoculated with the bacterial isolates and challenged with *B. cinerea* and *P. syringae* as described in previous sections. Leaves obtained from plants derived from non-inoculated seeds were used as controls.

2.11. Statistical analysis

Statistical analysis of the data was conducted using GraphPad Prism version 6.00 for Windows, GraphPad Software (La Jolla, CA, USA). A completely randomized design was used for all experiments and the number of replicates used

in each experiment was indicated in figure legends. With the exception of growth promotion experiments, results were subjected to analysis of variance (ANOVA) and means compared by Dunnett's test. Analysis of fresh weight of tomato plants inoculated with different isolates was performed by Student's t-test.

3. Results

3.1. Isolation of bacteria from inner tissues of tomato leaves, estimation of their taxonomic identity and potential for endophytic colonization

Ten isolates were obtained as described in Materials and Methods and their taxonomic identity was assessed by comparing 16S rDNA sequences with the Gen Bank database and reference strains. The results obtained by analyzing the highest scores of comparisons with the Gen Bank database are presented in Table 1 and were consistent with the clustering evidenced by phylogenetic trees (Fig. S1A). Some of the isolates clustered with the genera *Staphylococcus* (BT5), *Exiguobacterium* (GT4, BT3, and MT8) and *Bacillus* (MT3), all of which belong to the class Bacilli, Phyllum Firmicutes. The remaining isolates clustered with strains of the genus *Pseudomonas* (BT2, BT4, NT2 and NT3) and *Pantoea* (NT6), which belong to the class Gammaproteobacteria (Phyllum Proteobacteria) (Fig. S1A). Thus, the isolation of a significant proportion of Gammaproteobacteria is consistent with the previously reported abundance of this bacterial class in the endophytic community of tomato leaves by 16S ribosomal RNA gene pyrosequencing [14]. It is also worth keeping in mind that the culture medium and growth conditions used in the present work for bacterial isolation are expected to introduce a bias towards fast-growing mesophylic bacterium, which is consistent with the putative taxonomic identity of the isolates obtained.

Isolates BT3 and MT8 were closely related to *Exiguobacterium aurantiacum*, while GT4 showed 99% identity with an *Exiguobacterium* strain not identified at the species level (Table 1, Fig. S1A). Isolates BT5 and NT6 clustered with *Staphylococcus xylosus* and *Pantoea eucalypti*, respectively (Fig. S1A).

Isolate MT3 showed a 99% identity to both *B. amyloliquefaciens* and *B. methylotrophicus* (Table 1), and clustered with strains of the genus *Bacillus* (Fig. S1A). A more detailed phylogenetic tree constructed with several *Bacillus* strains allowed determining that this isolate is closer to *B. methylotrophicus* than to *B. amyloliquefaciens* (Fig. S1C). The 16S rDNA sequence of BT2 was highly similar to *Pseudomonas rhodesiae*, while NT2 was similar to *Pseudomonas veronii* (99% identity). NT3 and BT4 showed a high level of identity (99%) with different *Pseudomonas* strains not identified at the species level (Table 1). In order to have a more precise estimation of the taxonomic identity of these isolates, a phylogenetic tree was built with several reference strains of *Pseudomonas* spp. As shown in Fig. S1B, BT2 clustered with *P. rhodesiae* strains, while both BT4 and NT2 clustered with *P. veronii* and NT3 with *P. cichorii*.

The ability of the above-mentioned isolates to endophytically colonize tomato plants was analyzed by inoculation on seeds and further re-isolation from roots and leaves of plants grown under axenic conditions. Bacterial growth was detected in root pieces of plants inoculated with all isolates analyzed. In this case, bacterial growth initiated at the cut ends, but not on the external surface of root sections. Although the possibility exists that these results are due to the presence of bacterial remains on the root surface close to the cut ends, they suggest that all isolates were able to endophytically colonize roots. Isolates BT2, BT4, MT3, MT8, NT2 and NT6 were also recovered after plating leaf extracts, which rendered bacterial titers that ranged from 10^3 to10^4 CFU mg^{-1} leaf fresh weight (FW). BOX-PCR profiles (Section 2.3) of the bacterial colonies obtained after plating leaf extracts confirmed that the re-isolated bacteria were actually those previously inoculated on seeds (data not shown). These results strongly suggest that these isolates were able to endophytically colonize leaves after seed inoculation. Importantly, no bacterial growth was observed in leaf extracts and root sections obtained from control plants inoculated with 0.01 mol l^{-1} $MgCl_2$ pH 7.0, thus confirming the axenic condition of the plants used in this assay.

3.2. Antagonistic effects of bacterial isolates from tomato leaves towards phytopathogenic microorganisms in vitro

As an initial approach to evaluating the potential of bacterial endophytes for biocontrol of tomato diseases caused by

Table 1
Identification of bacterial isolates obtained from tomato leaves by 16S rRNA gene sequence analysis.

Isolate	Closest match in NCBI database (Accession number)	Identity (%)
BT2	*Pseudomonas rhodesiae* (KF054779.1)	98
BT3	*Exiguobacterium aurantiacum* (KJ722475.1)	100
BT4	*Pseudomonas sp.* (JF901709.1)	99
BT5	*Staphylococcus xylosus* (KC790245.1)	99
GT4	*Exiguobacterium sp.* (KM585592.1)	99
MT3	*Bacillus amyloliquefaciens/methylotrophicus* (KM488322.1/KM659226.1)	99/99
MT8	*Exiguobacterium aurantiacum* (KJ722475.1)	99
NT2	*Pseudomonas veronii* (JQ317805.1)	99
NT3	*Pseudomonas sp.* (JX067736.1)	99
NT6	*Pantoea eucalypti* (KF003411.1)	99

Table 2
In vitro inhibition of growth of phytopathogenic microorganisms by bacterial isolates obtained from tomato leaves.

Growth inhibition		
Isolate	*Botrytis cinerea* (%)[a]	*Pseudomonas syringae* (halo area[b]/colony area[c])
BT2	11.3 ± 2.9***	–
BT3	–	–
BT4	23.0 ± 0.8***	5.4 ± 0.4
BT5	–	–
GT4	3.8 ± 2.2	–
MT3	52.7 ± 3.0***	2.2 ± 0.3
MT8	–	–
NT2	25.2 ± 2.8***	5.7 ± 0.5
NT3	1.6 ± 0.5	–
NT6	29.1 ± 4.1***	–

[a] Inhibition of mycelial growth was calculated by comparing the diameter of colonies confronted with bacterial antagonists and control colonies, as described in Materials and Methods. Results are means of 3 replicate plates ± SE and statistical differences between plates inoculated with bacterial antagonists and controls are indicated as ***, $P \leq 0.001$.

[b] Area of the inhibition zone of *P. syringae* growth around the colonies of antagonistic bacteria.

[c] Area of the colony of the antagonistic bacterium. Results are the mean of three replicates ± SE.

B. cinerea and *P. syringae pv. tomato*, dual cultures of these pathogens and endophytic bacteria isolated from tomato leaves were performed. BT2, BT4, MT3, NT2 and NT6 inhibited mycelial growth of *B. cinerea* in vitro. BT4, MT3 and NT2 also inhibited in vitro growth of *P. syringae* pv. *tomato* (Table 2). Thus, BT4, MT3 and NT2 were able to inhibit in vitro growth of the two phytopathogenic microorganisms analyzed in the present work.

3.3. Protection of tomato plants against B. cinerea and P. syringae

Results obtained in the preceding section demonstrated that some of the bacterial isolates analyzed in the present work exert antagonistic effects towards *B. cinerea* and *P. syringae* pv. *tomato* in vitro. In order to determine whether such isolates are able to exert similar effects in planta, their ability to protect tomato plants against leaf infection by the above mentioned pathogens was analyzed by infiltrating detached leaves with the bacterial isolates immediately prior to inoculation with *B. cinerea* and *P. syringae* pv. *tomato*. In the case of leaves challenged with *B. cinerea*, disease severity was estimated on the basis of lesion size at different times after inoculation. Infiltration with NT2 caused a 56, 80 and 89% reduction in disease severity when evaluated 48, 72 and 96 h after inoculation (HAI), respectively (Fig. 1). BT2 and NT6 also showed a protective effect, which was evident from 72 HAI. BT2 reduced disease severity by 84 and 93% at 72 and 96 HAI, respectively, while the decrease provoked by NT6 at these two times after inoculation reached 56 and 58% (Fig. 1). As opposed to the previously mentioned isolates, infiltration with MT3 increased (≈1-fold) the severity of leaf damage caused by *B. cinerea* 72 and 96 HAI (Fig. 1). BT4 inoculation

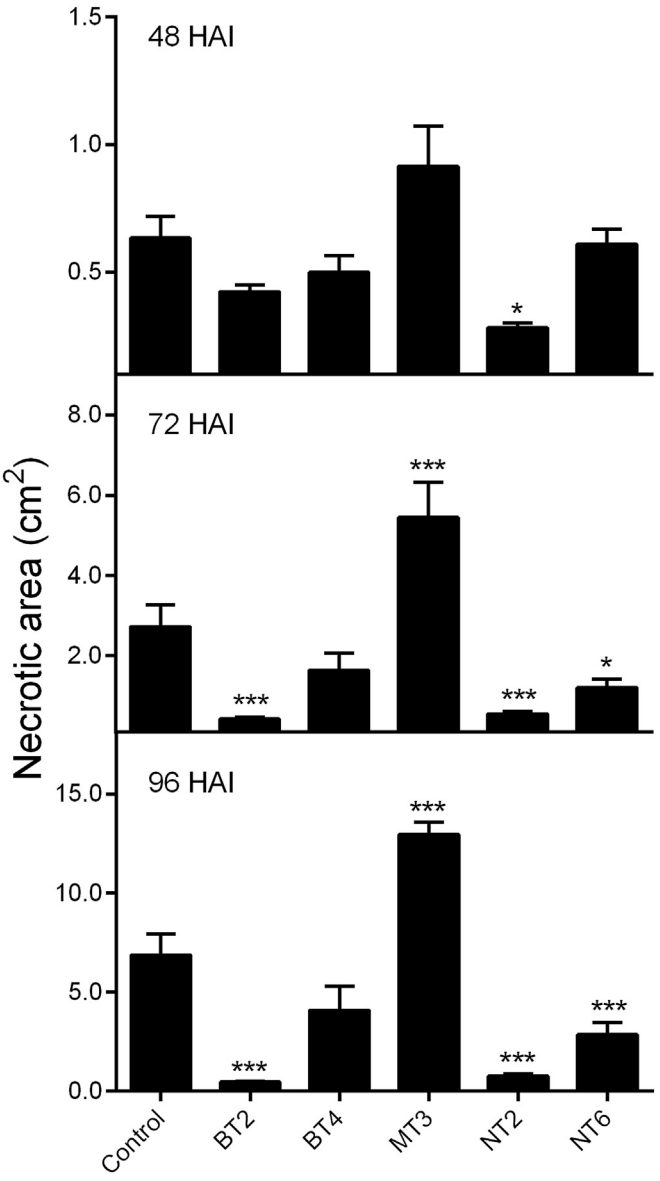

Fig. 1. Necrotic lesions provoked by *B. cinerea* infection of tomato leaves infiltrated with bacterial isolates obtained from leaves of field-grown tomato plants. Leaflets were inoculated with a 5-µl aliquot of a suspension of *B. cinerea* conidia (5×10^4 conidia ml^{-1}). The size of the necrotic area around the inoculation site was determined 48, 72 and 96 HAI using Image-ProPlus V 4.1 software. Results are means of 10 replicate leaflets ± SE, and statistically significant differences in the size of the necrotic area at each time between treatments and control are indicated as: *, $P \leq 0.05$; ***, $P \leq 0.001$.

exerted no effect on disease severity (Fig. 1). Leaf infiltration with BT4 and NT2 reduced *P. syringae* pv. *tomato* propagation at all post-inoculation times analyzed (Fig. 2). On the contrary, no changes were detected in the propagation of this pathogenic bacterium in leaves infiltrated with MT3 at any time post-inoculation (Fig. 2).

Those bacteria that proved to exert antagonistic effects towards *B. cinerea* and *P. syringae* in vitro were also tested for their ability to systemically protect tomato plants against leaf infection by these pathogens. For this purpose, an approach based on seed inoculation with candidate bacteria at the time

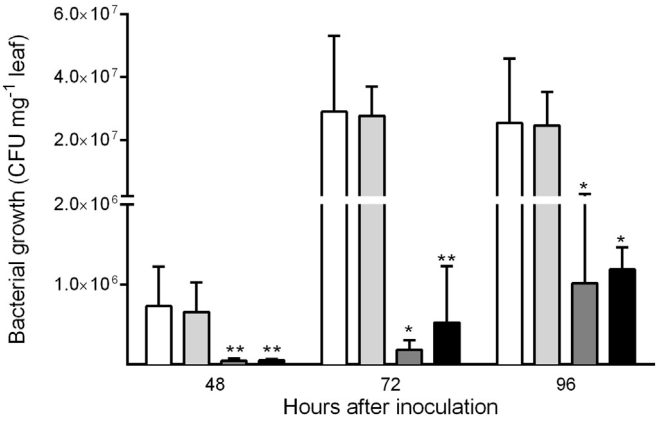

Fig. 2. Propagation of *P. syringae* pv. *tomato* in tomato leaves infiltrated with bacterial isolates obtained from leaves of field-grown tomato plants. Leaflets were detached from 6 week-old tomato plants, infiltrated with suspensions of isolates MT3 (▒), BT4 (■), NT2 (▒) and then were sprayed with a suspension of *P. syringae* pv. *tomato*. Leaflets infiltrated with $MgCl_2$ were used as controls (▭). *P. syringae* titers were determined 48, 72 and 96 HAI. Results are means of five replicates ± SE and statistically significant differences in bacterial titers at each time between treatments and control are indicated as: *, $P \leq 0.05$; **, $P \leq 0.01$.

of sowing and re-inoculation of stem bases 10 days after germination was used. Six weeks later, leaves were detached from plants and inoculated with *B. cinerea* or *P. syringae* pv. *tomato*. Inoculation with isolates BT4 and NT2 attenuated the development of lesions caused by *B. cinerea* infection. The protective effect observed with NT2 was evident at 48 and 72 HAI (Fig. 3), whereas with BT4, this effect was observed 72 HAI and continued until 96 HAI (Fig. 3). The rest of the isolates analyzed did not affect the development of lesions produced by *B. cinerea*. For plants challenged with *P. syringae* pv. *tomato*, inoculation with bacterial endophytes on seeds at sowing caused no reduction in the propagation of this pathogen (data not shown).

3.4. Production of antimicrobial compounds by bacterial isolates obtained from tomato leaves

Bacterial endophytes that antagonized *B. cinerea* and *P. syringae* in vitro were analyzed for their ability to excrete antimicrobial compounds into the culture medium. Cell-free filtrates from cultures of all isolates tested showed antifungal activity, by inhibiting germination of *B. cinerea* conidia (Fig. 4A). NT2 and MT3 filtrates caused the highest inhibition (≈70%), while BT4 filtrates reduced the percentage of germination by 56% (Fig. 4A). On the contrary, filtrates obtained from BT2 and NT6 showed the lowest inhibition of *B. cinerea* conidia germination (33 and 28% respectively). To evaluate the production of antibacterial compounds by BT4, MT3 and NT2, which showed the ability to inhibit growth of *P. syringae* (Table 1), supernatants were obtained from these isolates and their effect on growth of *P. syringae* was evaluated in vitro. Supernatants obtained from the three isolates inhibited growth of *P. syringae* when used at 50% (Fig. 4B).

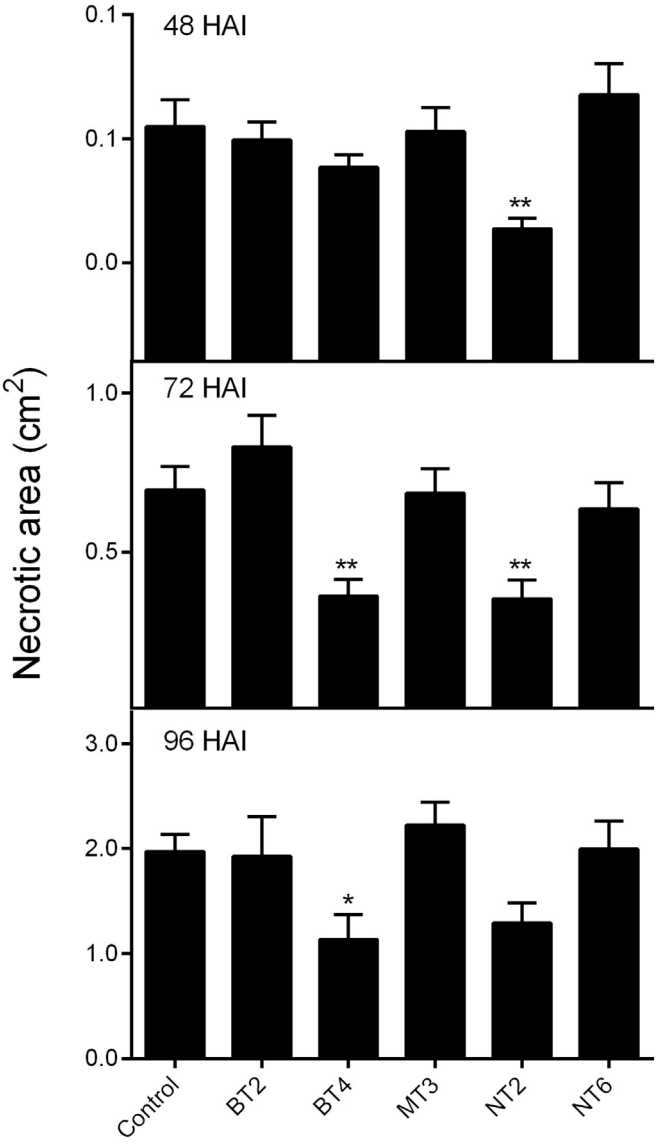

Fig. 3. Necrotic lesions provoked by *B. cinerea* infection of leaves of tomato plants grown from seeds inoculated with bacterial isolates obtained from leaves of field-grown tomato plants. Tomato leaflets were detached from 6-week-old plants inoculated with bacterial isolates at the time of sowing. Leaflets were inoculated with a 5-µl aliquot of a suspension of *B. cinerea* conidia (5×10^4 conidia ml^{-1}). The size of the necrotic area around the inoculation site was determined 48, 72 and 96 HAI using Image-ProPlus V 4.1 software. Results are means of 10 replicate leaflets ± SE and statistically significant differences in size of the necrotic area at each time between treatments and control are indicated as: *, $P \leq 0.05$; **, $P \leq 0.01$.

Supernatant of MT3 retarded growth of *P. syringae* even when tested at a 10% concentration (Fig. 4B).

3.5. Plant growth promotion, nutrient solubilization and phytohormone production

In order to determine if the endophytic community of tomato leaves harbors bacteria with potential for use as biofertilizers, the ten bacterial isolates initially identified at the taxonomic level were tested for their ability to promote growth of tomato plants. No symptoms of pathogenic effects were

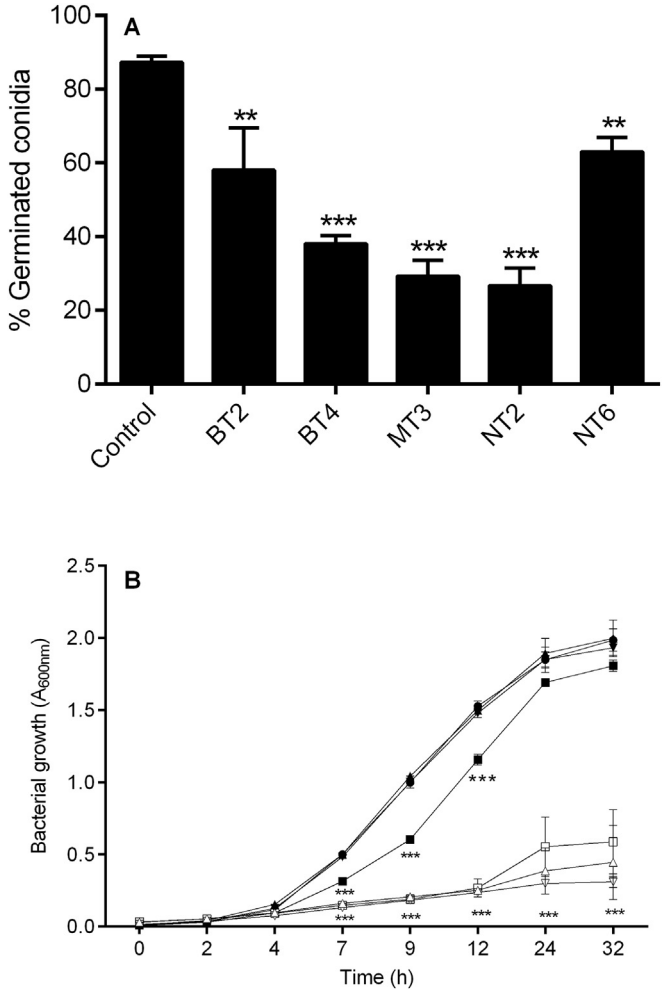

Fig. 4. Antimicrobial activity of cell-free filtrates prepared from cultures of bacterial isolates obtained from leaves of field-grown tomato plants. (A) Germination of *B. cinerea* conidia in the presence of cell-free filtrates obtained from cultures of bacterial isolates from tomato leaves. Twenty-µl-aliquots of a suspension of *B. cinerea* conidia (1.5×10^4 conidia ml^{-1}) were mixed with an equal volume of cell-free filtrates obtained from liquid cultures of isolates BT2, BT4, MT3, NT2 and NT6 and were incubated for 16 h. Conidia incubated with TSB were used as controls. The percentage of germinated conidia was calculated after analyzing a total of 120 conidia per well. Results are means ± SE of four replicate wells and statistical differences in the percentage of germinated conidia between treatments and controls are shown as: **, $P \leq 0.01$; ***, $P \leq 0.001$. (B) Growth of *P. syringae* pv. *tomato* in the presence of cell-free filtrates obtained from cultures of bacterial isolates obtained from tomato leaves. Liquid cultures of *P. syringae* pv. *tomato* (3 ml, DO = 0.01) were mixed with cell-free culture filtrates obtained from liquid cultures of bacterial isolates, to a final concentration of 10 or 50% v/v (–■–,10% MT3; –□–, 50% MT3; –▼–, 10% NT2; –▽–, 50% NT2; –▲–, 10% BT4; –△–, 50% BT4). *P. syringae* cultures mixed with TSB were used as controls (–●–). Growth of *P. syringae* was evaluated according to the absorbance at 600 nm for 32 h. Results are means ± SE of three replicates and statistical differences between treatments and controls are shown as: ***, $P \leq 0.001$.

detected in plants inoculated with any of them. Plants inoculated with isolates BT4, BT5, NT6 and MT3 exhibited an increase in fresh weight (FW) of stems as compared to non-inoculated plants (Table 3). Among these isolates, NT6 and MT3 also caused an increase in root FW. Isolate BT2 only promoted root growth. The remaining isolates caused no significant effects on plant growth (Table 3).

Bacterial isolates were also evaluated for their ability to solubilize non-soluble P and siderophore production. Four isolates (BT2, BT4, NT2 and NT6) showed positive results for both assays, while an additional isolate (MT3) only solubilized P (Table 3). Among the above-mentioned isolates, BT2 and BT4 showed the highest P solubilization and siderophore production. The remaining isolates were not able to solubilize nutrients (Table 3).

The four bacterial isolates that promoted growth of tomato plants were tested for their ability to produce phytohormones. IAA production was tested both with and without the addition of the IAA precursor Trp to the culture medium. All these isolates produced IAA, three of them (BT2, BT4, and MT3) in the range of 36.3–56.3 µg ml^{-1}. Isolate NT6 produced a higher concentration of IAA than the other isolates, reaching 253.8 µg ml^{-1} (Table 3). Trp addition to the culture medium significantly increased IAA production by all four isolates. This treatment increased IAA by 56-, 16-, 20- and 131-fold for isolates BT2, BT4, MT3 and NT6, respectively. Thus, under this condition, isolate NT6 again produced a much higher IAA concentration than the remaining three isolates (Table 3). Other phytohormones such as the cytokinin zeatin, ABA and GA_3, were also detected, although at very low and highly variable concentrations (data not shown).

4. Discussion

Current knowledge of the potential of bacterial species for plant growth promotion and protection against pathogens derives, to a large extent, from the study of rhizospheric microorganisms. However, information on the diversity and abundance of beneficial microorganisms in aboveground plant organs is less abundant [27]. In this regard, bacteria capable of endophytically colonizing inner plant tissues from aerial organs have the potential to compete with pathogens that invade and colonize such organs as part of their pathogenic strategies [28]. Conditions for bacterial propagation and survival in inner plant tissues are more stable than in the phyllosphere, mainly in terms of water and nutrient availability. Thus, it is not surprising that the communities of endophytic bacteria of tomato leaves show a particular composition, as analyzed by culture-independent methodologies [14]. On this basis, the present work aimed to isolate and identify potentially endophytic bacteria from tomato leaves capable of protecting this organ against infection by pathogenic microorganisms and, in turn, exert beneficial effects on plant growth. As a consequence, a limited number of isolates (ten) obtained from surface disinfected leaves were first characterized at the taxonomic level prior to further analysis of their biocontrol and growth-promotion potential. Evidence concerning the ability to endophytically colonize aerial organs of tomato plants was obtained for six of these isolates (BT2, BT4, MT3, MT8, NT2 and NT6) after seed inoculation and re-isolation from leaf extracts. The fact that the remaining four isolates could not be recovered from leaf extracts of seed-inoculated

Table 3
Plant-growth promoting traits of bacterial isolates obtained from tomato leaves.

Isolate	Fresh weight roots (g)[a]	Fresh weight shoots (g)[a]	Phosphate solubilization[b]	Siderophore production[b]	IAA (μg ml^{-1}) -Trp[c]	IAA (μg ml^{-1}) +Trp[c]
Control	1.5 ± 0.4	4.4 ± 0.9				
BT2	2.3 ± 0.4*	6.5 ± 2.7	12.7 ± 1.6	10.1 ± 3.4	36.3 ± 13.1	2015.1 ± 716.8
BT3	2.6 ± 1.0	6.2 ± 2.3	–	–	NA	NA
BT4	2.8 ± 1.0	7.8 ± 1.7*	11.9 ± 3.2	14.6 ± 1.3	55.3 ± 20.3	870.6 ± 242.4
BT5	1.9 ± 0.4	8.7 ± 1.7*	–	–	NA	NA
GT4	2.2 ± 1.0	6.6 ± 2.0	–	–	NA	NA
MT3	3.3 ± 0.4**	8.3 ± 1.8*	1.9 ± 0.3	–	38.3 ± 12.5	747.4 ± 355.6
MT8	1.9 ± 0.7	6.0 ± 2.0	–	–	NA	NA
NT2	1.4 ± 0.5	6.2 ± 1.4	10.0 ± 3.8	12.9 ± 0.1	NA	NA
NT3	2.3 ± 1.5	7.3 ± 2.3	–	–	NA	NA
NT6	2.8 ± 0.2**	8.3 ± 2.1*	2.1 ± 0.3	4.6 ± 0.5	253.8 ± 57.8	33,269.1 ± 22,982.0

[a] Fresh weight of roots and shoots was measured on 6-week-old tomato plants inoculated with bacterial isolates at sowing. Non-inoculated plants were used as controls. Results are means of four biological replicates ± SE and statistical differences between inoculated and control plants are shown as *, $P \leq 0.05$; **, $P \leq 0.01$.

[b] Phosphate solubilization and siderophore production are shown as the ratio between the area of the solubilization or siderophore production halo and the area of the colony of each isolate. Results are means of three replicates ± SE.

[c] IAA was determined in the supernatants of 48 h-old cultures of bacteria grown in AB medium with or without the addition of the IAA precursor Trp (0.75 mmol l^{-1}). Results are means of four replicates ± SE. NA = Not analyzed.

plants raises several possible explanations for their presence in leaf extracts initially obtained from leaf-grown tomato plants. First, it is possible that these isolates are actually able to endophytically colonize leaves, but that the root is not the entry path to the plant. Alternatively these isolates could have poor efficiency for colonization of aerial organs after getting into the plant through the root system. Another possibility is that the endophytic colonization of leaf tissues by certain strains requires cooperative interaction between them. Finally, some of the strains found to be unable to colonize aerial organs after seed inoculation under axenic conditions could have been present as epiphytes on leaf surfaces of field-grown plants initially used as a source of bacteria isolation, and may have resisted the surface disinfection procedure. In this case, these isolates would actually represent epiphytic and not endophytic bacterial strains. In any case, it is worth to keeping in mind that, as will be discussed below, the isolates that showed interesting features related to biocontrol and plant-growth promotion were mainly those that proved to be able to colonize leaves after seed inoculation in the above-mentioned tests.

Among the ten isolates obtained in the present work, three of them were closely related to *Exiguobacterium* spp. reference strains according to their 16S rDNA sequences. The complete genome sequence of *Exiguobacterium* sp. MH3, a strain obtained from the rhizosphere of duckweed (*Lemna minor*), has recently been published [29], but reports on the endophytic habit of bacteria belonging to this genus are not abundant. In this regard, *Exiguobacterium* spp. were detected as stem endophytes of potato (*Solanum tuberosum*) plants infected by *Erwinia carotovora* subsp *atroseptica* [30]. The present work thus provides evidence on the ability of isolates closely related to *Exiguobacterium* spp. to colonize other plant species, although isolates hereby characterized showed no interesting traits related to growth promotion or disease control, as described in the paragraphs below.

An isolate closely related to *S. xylosus* was also found to be a member of the microbial community of tomato leaves in this study (BT5). *S. xylosus* is one of the main components of the phyllospheric community of spinach (*Spinacia oleracea*) leaves during storage [31] and was also detected in the rhizosphere of *Mazus* spp. plants [32], but to our knowledge this bacterium was not reported to be a component of endophytic communities. On this basis, and also taking into account that BT5 could not be recovered from inner leaf tissues of axenically grown plants inoculated at sowing, it is not possible to confirm that isolate BT5 is actually endophytic on tomato leaves.

Other isolates were related to phylogenetic taxa more frequently associated with plants. This is the case for MT3 and NT6, isolates related to *Pantoea eucalypti and Bacillus methylotrophicus*, respectively. *Pantoea* spp. were previously found to be associated with tomato plants, in which they were detected in leaf extracts, as well as on leaf and fruit surfaces [33–36]. *Bacillus* spp. are ubiquitous in rhizospheric, phyllospheric and endophytic microbial communities associated with plants [37]. However, *B. methylotrophicus* has been proposed to be a novel species relatively recently, after its isolation from the rhizosphere of rice [38]. Thus, information on its interaction with plants is still scant compared to other *Bacillus* spp. However, *B. methylotrophicus* strains were detected in the endosphere of apple (*Malus domestica*) [39] and banana (*Musa* spp.) plant roots [40] and *Salvia miltiorrhiza* plants [41]. The present work thus provides evidence of the ability of isolates closely related to *B. methylotrophicus* to colonize tomato plants and also exert beneficial effects on them, as discussed in further paragraphs.

Pseudomonas spp. were also identified in the present work as components of the bacterial community of tomato leaves. This genus of Gammaproteobacteria comprises many species that interact with plants in different ways, exerting beneficial, neutral or detrimental effects on their hosts. Thus, many *Pseudomonas*

spp. are well known PGPRs [42], while others cause severe plant diseases [43]. However, some *Pseudomonas* spp. closely related to certain isolates identified in the present work have been less well studied in this regard. *P. cichorii*, a species to which isolate NT3 was closely related, has been reported to be a pathogen on a variety of plant hosts, causing necrotic lesions on leaves and shoots [43]. However, tomato plants inoculated with NT3 in the present work did not show disease symptoms or deleterious effects on growth. Isolates related to *P. veronii* (BT4 and NT2) and *P. rhodesiae* (BT2) were also represented in the bacterial collection characterized in the present work. These two species were previously reported as poplar (*Populus trichocarpa*) endophytes [44], and *P. veronii* was also found as a rice (*Oryza sativa*) endophyte [45]. *P. rhodesiae* was also isolated from surface-disinfected pepper (*Capsicum annuum*) shoots [46] and was reported to be a shoot endophyte of *Oxalis corniculata* [47]. The antagonistic potential towards pathogens and the growth-promoting effects of *Pseudomonas* isolates obtained in the present work are discussed below.

4.1. Antagonistic effects and biocontrol potential of bacterial endophytes towards phytopathogenic microorganisms

Leaf infiltration experiments demonstrated that colonization of inner tissues of tomato leaves by BT2, NT2 and NT6 provides some level of protection of this organ against *B. cinerea* infection. It is worth bearing in mind that this experiment involved simultaneous inoculation of potentially antagonistic bacteria and *B. cinerea*. Thus, the experimental conditions probably did not provide enough time for activation of host defense mechanisms prior to inoculation with the pathogenic fungus. On this basis, and also taking into account the fact that the antagonistic bacteria were directly inoculated into the same organ subsequently challenged with the pathogen, it can be speculated that direct competition and antibiosis played an important role in the antagonistic effects exhibited by BT2, NT2, and NT6. Results obtained after leaf infiltration with isolate MT3 in turn demonstrated that in spite of being per se innocuous for the plant host, some bacteria could potentiate the deleterious effects of pathogen infection, thus highlighting the importance of a meticulous evaluation of potential biocontrol agents under different experimental conditions. Leaf infiltration experiments also demonstrated that leaf colonization by NT2 has the potential to protect tomato leaves not only against a typical necrotroph like *B. cinerea*, but also against pathogens such as *P. syringae*, which deploys a biotrophic strategy of pathogenesis during early stages of infection.

The biocontrol potential of *Pseudomonas* spp. such as *P. fluorescens* has been clearly established [48,42], but *P. veronii* and *P. rhodesiae* have been less well studied in this regard [49,50]. Similarly, *Pantoea eucalypti* has not been studied as a biological control agent. Results hereby presented suggest that the potential of isolates closely related to *Pseudomonas* and *Pantoea* spp. characterized in the present work warrant further study as potential biological control agents of tomato diseases.

Additional experiments that involved seed inoculation with bacterial isolates obtained in the present work and further inoculation of leaves with *B. cinerea* six weeks later enabled determining that isolates BT4 and NT2 had the potential to systemically protect tomato plants against this fungus, thus being interesting candidates as biocontrol agents of diseases caused by *B. cinerea*. It is worth pointing out that isolates such as BT2 and NT6 induced no systemic protection against *B. cinerea* in spite of the local protective effect provided by their infiltration into leaves. Thus, it is possible that these isolates are capable of antagonizing *B. cinerea* only by competition or antibiosis when they are present in tissues targeted by the pathogen. On the contrary, BT4 and NT2 could protect host plants through activation of host defenses, a hypothesis that deserves further analysis.

4.2. Antimicrobial compounds produced by bacterial endophytes

Evaluation of the antimicrobial activity of cell-free supernatants obtained from cultures of bacterial isolates that were found to antagonize *B. cinerea* (BT2, BT4, MT3, NT2 and NT6) and *P. syringae* (BT4, MT3 and NT2) in dual cultures revealed that some of these isolates are able to produce antifungal and antibacterial compounds, at least under in vitro conditions. Thus, it is possible that these compounds play a role in antagonism towards *B. cinerea* and *P. syringae* in tomato plants. Conidia are the primary source of inoculum for diseases caused by *B. cinerea* on aerial plant organs. In planta propagation is also of key importance for the pathogenesis process of *P. syringae*. Thus, the ability to inhibit germination of *B. cinerea* conidia and *P. syringae* multiplication could contribute to control of these pathogens by the above-mentioned isolates. The isolates found to produce antifungal compounds were closely related to *P. rhodesiae* (BT2), *P. veronii* (BT4 and NT2), *B. methylotrophicus* (MT3) and *P. eucalypti* (NT6). *B. methylotrophicus* is known to produce phenaminomethylacetic acid [51] and other yet unidentified antifungal compounds [52]. *P. rhodesiae* and *P. veronii* are also known to antagonize fungal pathogens [46,48,50,53,54], but no antifungal compounds have been identified from these particular *Pseudomonas* spp. Thus, further characterization of antimicrobial compounds produced by the above-mentioned isolates obtained in the present and previous works would contribute to identifying novel bioactive molecules involved in antagonistic interactions between potential biological control agents and pathogenic microorganisms.

4.3. Growth promotion, nutrient solubilization and phytohormone production by bacterial endophytes

Seed inoculation of the ten isolates characterized in the present work revealed that several of them (BT2, BT4, BT5, MT3 and NT6) are able to promote growth of tomato plants. For growth promotion assays performed in this work, plants were irrigated with nutrient solutions containing non-limiting

levels of soluble nutrients. Thus, growth promotion mediated by the above-mentioned bacteria is not expected to result from improved nutrient availability. More probably, growth promotion resulted from the release of growth-promoting compounds into the growth medium by these bacteria. In this regard, four of these strains, which were tested for production of plant growth regulators, were all found to produce the auxin IAA, which is produced by many PGPRs [55]. This finding suggests that IAA could participate in the growth-promotion effects of the above-mentioned bacteria, although the contribution of other growth-promoting compounds cannot be ruled out. Moreover, the several-fold increase in IAA levels induced by tryptophan strongly suggests that these bacteria produce IAA through the tryptophan-dependent pathway [55]. In addition, it was also interesting to identify isolates able to solubilize inorganic P sources (BT2, BT4, NT2, NT6 and MT3) and produce siderophores (BT2, BT4, NT2 and NT6). The well-known relation of these physiological traits with plant growth promotion and growth-promoting ability of *Bacillus, Pseudomonas* and *Pantoea* spp. [56–58] make these isolates interesting candidates for formulation of biofertilizers and/or biostimulators, based either on individual isolates or on a combination of them.

Finally, it is worth pointing out that isolates BT2, BT4, MT3, NT2 and NT6 analyzed in the present work exhibited a combination of beneficial traits related to biocontrol and growth promotion activities. This finding is interesting not only because of the potential of these bacteria for formulation of biocontrol agents and biofertilizers/biostimulators, but also because it may help to elucidate the genetic basis for the presence of multiple beneficial traits associated with growth promotion and biocontrol in their genomes.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2015.11.001.

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