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Evaluation of the Growth-Promoting Potential of Endophytic Bacteria Recovered from Healthy Tomato Plants

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Abstract

The study examined the plant growth-promoting potential of 38 endophytic bacterial isolates recovered from healthy tomato plants. Eight isolates were selected as the most efficient in improving the plant height by 30.5-35.9%, the aerial part fresh weight by 47.4 -56.9%, the maximum root length by 17.3- 28.3%, and the root fresh weight by 44.7-58.8% compared to control. They were morphologically and biochemically characterized and identified using 16S rDNA sequencing genes as *Stenotrophomonas maltophilia* CT12, *S. maltophilia* CT13, *S. maltophilia* CT16, *Pseudomonas geniculata* CT19, *B. amyloliquefaciens* CT32, *B. subtilis* subsp. *inaquosorum* CT43, *B. licheniformis* SV4, and *B. subtilis* SV5. All isolates were shown able to produce indole-3-acetic acid and displayed pectinolytic activity. Phosphate solubilization ability was recorded in *S. maltophilia* CT13, *S. maltophilia* CT16, *B. subtilis* subsp. *inaqosorum* CT43 and *B. licheniformis* SV4. This study demonstrates that healthy tomato plants may be naturally colonized by beneficial endophytic bacteria with growth-promoting potential useful for the improvement of tomato growth.

Keywords: Characterization; Endophytic bacteria; Metabolites; Plant growth-promoting ability; Tomato

Introduction

Tomato (*Solanum lycopersicum* L.) is widely grown worldwide and of major importance for agricultural industry [1]. Genetics, genomics, and breeding are well widely investigated but this species was not sufficiently valorized as natural source of biocontrol and/or biofertilizing agents [2]. Searching beneficial microbial communities naturally associated to tomato may contribute to the identification of potential candidates with plant growth-promoting traits.

Although the majority of research studies undertaken on plant-associated bacteria have been focused on rhizobacteria, the use of endophytic bacteria as new and promising alternative for the improvement of plant growth and health is becoming of increasing interest [3]. Their increased importance is justified by the fact that once reintroduced inside plant tissues, a relationship can be established between them and their host plants. This relationship is considered more stable than that undertaken with rhizospheric and/or epiphytic bacteria [4]. Next, endophytes may remain at their entry points or spread throughout the plant tissues without causing any harmful effects on their host. They can be isolated from inner plant parts or from surface-disinfested plant tissues of various organs [5].

Endophytic bacteria are ubiquitous in various cultivated plant species such as *Capsicum annum* [6], *Solanum tuberosum* [7], and other non solanaceous species [8-12]. They are involved in plant growth-promotion and/or biocontrol suppression of associated diseases [4].

Several previous studies demonstrated the plant growth-promoting effect induced by endophytic bacteria. In fact, various bacteria, recovered from sugar cane roots and stems, were shown able to enhance the growth of this plant [13]. Also, four endophytic bacteria, namely *Azospirillum brasilense*, *Burkholderia ambifaria*, *Gluconacetobacter diazotrophicus*, and *Herbaspirillum seropedicae* were found capable to colonize root, stem and leaf tissues of *S. lycopersicum* var. *lycopersicum* and to enhance its growth [14].

Plant Growth-Promoting Bacteria (PGPB) is able to promote plant growth either directly by facilitating the acquisition of essential nutrients [15] or by modulating the biosynthesis of phytohormones and lowering ethylene levels [16]. Indirectly, PGPB enhance plant growth by inhibiting pathogens and/or by inducing systemic resistance [17]. In fact, *Pseudomonas aeruginosa* HR7, *Pseudomonas* sp. TEP3, *Bacillus* sp. TEB6, *Klebsiella* sp. TEK1, *Citrobacter* sp. and *B. amyloliquefaciens* JK-SD002 are able to produce indole-3-acetic acid, salicylic acid, siderophores and organic acids and to solubilize phosphate. All these effects are known to be involved in growth promotion [18,19] even in low-fertility soils [20].

There is a lack of data on the diversity of beneficial endophytic bacteria naturally associated to tomato in Tunisia and their potential use as bio-fertilizing agents. Therefore, this study was carried out to evaluate the ability of 38 tomato-associated endophytic bacterial isolates to promote growth and to select the most ones to determine the mechanisms of action involved in this effect.

Materials and Methods

Plant material

Healthy tomato plants cv. Sahel® Syngenta (commercially cultivated hybrid resistant to Verticilium, Fusarium Wilt (Fol 1-2), Fusarium crown and root rot, grey leaf spot, tomato mosaic virus and root rot nematode) grown under greenhouse conditions were explored as potential source for isolation of endophytic bacteria. In order to collect isolates from different developmental stages, tomato plants were sampled in March, April, May and November 2013 from Teboulba (N35°38'38.256"; E10°56'48.458"), Chott-Mariem (N35°56'20.451"; E10°33'32.028") and Knaies (N35°40'59,999"; E10°31'0,001"), Tunisia.

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Collected samples were washed thoroughly with tap water before disinfection and isolation of endophytic bacteria.

Tomato cv. Rio Grande seeds were used for the *in vivo* bioassays. Seedlings were grown in alveolus plates (7 × 7 cm) filled with sterilized peat® (Floragard Vertriebs GmbH für gartenbau, Oldenburg) and maintained under greenhouse with 16 h photoperiod, 60-70% relative humidity and air temperatures ranging between 20 and 30°C. Seedlings were watered regularly until reaching the two-true-leaf growth stage. Seedlings with approximately similar heights were used for all the *in vivo* bioassays.

Isolation of endophytic bacteria and growth conditions

Collected tomato roots, stems, leaves, flowers, fruits and/or seeds (five samples each) were individually disinfected by soaking in 70% ethanol for 1 min, immersion in 1% sodium hypochlorite for 10 min and then in 70% ethanol for 30s. They were rinsed three times with sterile distilled water (SDW) and air-dried on sterile filter papers. Twenty pieces (0.5 cm in length) from each sampled organ were aseptically transferred on Nutrient Agar (NA) medium and incubated at 25°C for 48 h. The efficiency of surface sterilization process was checked according to Hallmann et al. [5].

For each plated organ, bacterial colonies exhibiting macro-morphological diversity were grown separately on NA medium and incubated at 25°C for 48 h. Total number of bacterial isolates recovered per organ and per sampling site are given in Table 1. Stock cultures were maintained at -20°C in Nutrient Broth (NB) supplemented with 40% glycerol. They were previously grown on NA and incubated at 25°C for 48 h before use in the different trials.

Test of hypersensitivity reaction and hemolytic activity

Bacterial isolates were checked for their hypersensitivity reaction on tobacco leaves according to Nawangsih et al. [21] method. After incubation for 24 h under ambient room conditions, isolates inducing the formation of chlorotic and/or necrotic zones on inoculated leaf areas were considered as phytopathogens and excluded from the following trials.

Isolates were also screened for their hemolytic activity on Blood Agar® (HiMedia, India) medium according to Murray et al. [22]. After incubation at 25°C for 48 h, isolates forming clear zones around their colonies were classified as pathogenic to humans and excluded from the following trials.

Test of the endophytic colonization potential

This test was performed for 63 bacterial isolates recovered from tomato cv. Sahel according to Chen et al. [23]. Isolates were grown on NA supplemented with streptomycin sulphate (100 µg ml^{-1} w v^{-1}) and rifampicin (100 µg ml^{-1} w v^{-1}). Isolates exhibiting resistance to both antibiotics were selected and the wild types were used for the inoculation of tomato cv. Rio Grande seedlings.

Seedlings were soaked for 30 min in a bacterial cell suspension (10^8 cells ml^{-1}) and controls were dipped in SDW only. Seedlings were transplanted into individual pots (12.5 × 14.5 cm) filled with sterilized peat. Five replicates of one seedling each were used for each individual treatment. The whole experiment was repeated twice. Tomato seedlings (inoculated and uninoculated) were grown under greenhouse conditions as described above. After 60 days of growth, stem sections (1 cm in length) were disinfected as described above and cut longitudinally then pierced with a sterile-nipper and the liquid exuding from the internal tissues was streaked on NA medium amended with both antibiotics (100 µg ml^{-1}) (w v^{-1}). After 48 h of incubation at 25 °C, isolates showing growing colonies similar to the wild type ones were considered as endophytes.

Test of the plant growth-promoting ability

Thirty eight bacterial isolates were assessed for their ability to enhance growth of tomato seedlings. Roots of tomato cv. Rio Grande seedlings were soaked for 30 min in a bacterial cell suspension (10^8 cells ml^{-1}) and the controls were treated similarly using SDW [14]. Seedlings were transferred to individual pots (12.5 × 14.5 cm) filled with sterilized peat. Five replicates of one seedling each were used for each individual treatment. The whole experiment was repeated twice. At 60 days post-planting, plant height, maximum root length and fresh weight of the aerial parts and roots were measured.

Characterization and Identification of the Most Efficient Plant Growth-promoting Agents

Morphological and biochemical characterizations

Morphological and biochemical characterizations were performed for eight selected isolates. Colonies were characterized macro-morphologically based on their form, margin, elevation, surface, opacity, and pigmentation on NA medium [18]. Gram's staining was performed using light microscopy. Biochemical characterization was performed using conventional tests such as catalase, urease, lecithinase, deaminase, nitrate reductase, lysine decarboxylase, Red of Methyl (RM), Vosges Proskauer (VP), mannitol, simmons citrate, indole, tryptophane, hydrogen sulfide, gas production, and pyocyanin on King A based on Schaad et al. [24].

Molecular identification

Identification of the eight bacterial isolates was performed according to van Soolingen et al. [25] protocol for Gram positive bacteria and Chen and Kuo [26] for negative Gram bacteria by sequencing of 16 rDNA gene and homology analysis after extraction of genomic DNA. The two universal eubacterial primers 27f and 1492r used for amplification of 16S rDNA gene are 5'-AGAGTTTGATC(A/C) TGGCTCAG-3' and 5'-TACGG(C/T)TACCTTGTTACGACTT-3', respectively [27]. Amplifications were carried out in Thermal Cycler® (CS Cleaver, Scientific Ltd., TC 32/80). PCR conditions were as follows:

Total number of isolates[a]	Number of isolates per sampling site	Number of isolates per organ	Sampling month
63	Chott-Mariem (30) Teboulba (18) Knaies (11) M'saken (4)	Root (3) Stem (20) Leave (18) Flower (10) Green fruit (7) Red fruit (3) Seed (2)	March 2013 (7) April 2013 (22) Mai 2013 (28) November 2013 (6)

Table 1: Number of endophytic bacterial isolates recovered from healthy tomato cv. Sahel plants. [a]Isolates exhibiting diversity in their macro-morphological traits on Nutrient Agar (NA) medium.

one denaturing cycle at 94°C for 4 min, followed by 40 denaturing cycles at 94°C for 30 s, annealing at 45°C for 30 s, and polymerization at 72°C for 45s. The amplification was terminated with a final extension cycle of 7 min at 72°C. The homology of the 16S rDNA sequence of a given isolate was performed using BLAST-N program from GenBank database (http://www.ncbi.nlm.gov/BLAST/). Alignment of the sequences was carried out using ClustalX (1.81). Phylogenetic analysis for the aligned sequences was performed using the Kimura two-parameter model [28]. The phylogenetic tree was constructed based on the neighbor joining (NJ) method with 1000 bootstrap sampling.

Test of seed germination stimulation

Tomato cv. Rio Grande seeds were superficially disinfected for 3 min in 5% sodium hypochlorite (NaOCl) and then rinsed six times with SDW. Ten disinfected seeds were separately placed on filter papers impregnated with SDW and placed in sterile Petri plates. Two hundred µL of bacterial cell suspensions (~10^8 cells ml^{-1}) were injected into each Petri plate contained 10 seeds. Seeds treated with a same volume of SDW were used as control. Two replications of ten seeds each were performed for each individual treatment. The whole experiment was repeated twice. Plates were incubated at 25°C in the dark. The percentage of germination was noted after 2 and 5 days of incubation [29].

Test of production of indole-3-acetic acid

Eight isolates were assessed for their ability to produce indole-3-acetic acid (IAA) based on Sgroy et al. [30] method. Isolates were cultured into LB medium amended with L-tryptophan (50 µg ml^{-1} w v^{-1}) under continuous shaking for 2 days in the dark. The negative control was an uninoculated LB- L-tryptophan medium. Treatments were performed in triplicate. Experiment was repeated twice. Absorbance was read daily at 530 nm. IAA concentration was determined using a standard curve prepared from IAA dilution series at 100 µg ml^{-1} w v^{-1} in LB medium.

Test of phosphate solubilization ability

Phosphate solubilization ability was evaluated qualitatively according to Sgroy et al. [30] where the colonies were individually deposited onto Pikovskaya agar medium. Uninoculated plates were used as control. Treatments were performed in triplicate. Experiment was repeated twice. After 7 days of incubation at 28 ± 2 °C, the clear zone formed around colonies was measured.

Test of pectinolytic activity

Eight bacterial isolates were assessed for their pectinolytic activity onto sterilized NA-pectin® (ICN Biomedicals, Inc, Germany) medium (0.5 % w v^{-1}) according to Tiru et al. [31]. Bacterial cell suspensions (~10^8 cells ml^{-1}) were streaked separately on NA-pectin medium amended plates. Control plates contained NA-pectin only. Three plates were used for each individual treatment. Each experiment was repeated twice. The presence of clear zones around the bacterial spots was checked after 48 h of incubation at 28 ± 2°C.

The pectinolytic activity was also determined quantitatively by measuring the amount of reducing sugars liberated from pectin using dinitrosalicylic acid (DNS) solution. The reducing sugar concentration was determined by optical density at 540 nm. The polygalacturonic acid was used as standard calibration. The concentration of polygalacturonic acid was derived from a standard curve prepared by a dilution series of polygalacturonic acid (1%) (w v^{-1}) in 50 mM phosphate buffer (pH 7.0). Each individual treatment was replicated thrice. The whole experiment was repeated twice. The pectinolytic activity was calculated using the following formula: *Activity* (U ml^{-1}) = (([SR] × RV)/Z) × 10^6 × (1/t) × (1/EV), where [RS]: reducing sugar concentration (g l^{-1}), RV: reactional volume (10^{-3} l), t: timing of reaction (30 min), EV: enzyme solution volume (0.5 ml), Z: molar mass of polygalacturonic acid liberated [32].

Statistical Analysis

Data were subjected to a one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) software for Windows version 16.0. *In vivo* trials were performed following a completely randomized design. Means were separated using Student Newman Keuls tests to identify significant pair-wise differences at $P \leq 0.05$. For the test of germination of tomato seeds, means were separated using test-t of Student at $P \leq 0.05$.

Results

Bacterial isolates recovered from tomato and their endophytic behavior

Sixty three (63) bacterial isolates, exhibiting diversity in their macro-morphological traits on NA medium, were recovered from healthy tomato cv. Sahel plants. Isolates were collected from four Tunisian geographical sites i.e. 30 isolates from Chott-Mariem, 18 from Teboulba, 11 from Knaies and 4 from M'saken. The number of isolates was higher in stems (31.8% of total isolates collected) than in leaves (28.6%), flowers (15.8%), fruits (15.8%), roots (4.8%), and seeds (3.2%) (Table 1). Of the 63 collected, 51 isolates were found to be resistant to streptomycin and rifampicin when grown on NA amended with both antibiotics. Challenged to tomato cv. Rio Grande seedlings, 38 isolates were successfully re-isolated from the internal stem tissues when plated on NA amended with both antibiotics (Table 2). They were classified as endophytes and retained for further screening of their plant growth-promoting potential.

Hypersensitivity reaction and hemolytic activity of selected endophytic isolates

No hypersensitive reaction (HR) and no hemolytic activity were detected after incubation. Thus, the selected endophytic isolates were found to be nonpathogenic to plants and humans, respectively, and were retained for the following tests (Table 2).

Plant growth-promoting potential of tomato-associated endophytic bacteria

All plant growth parameters (plant height, aerial part and root fresh weight, and maximum root length), noted 60 days post-treatment, revealed a significant ($P \leq 0.05$) variation in depending on tested bacterial treatments.

Aerial part development

A significant increase in plant height, by 30.5-35.9% compared to control, was noted on tomato plants treated with the isolates CT12, CT43, SV5, CT13, CT16, CT19, SV4, and CT32 (Table 3). The aerial part fresh weight was also significantly enhanced by 39.5- 57% over control following treatments with SV3, SV7, CT12, CT43, SV5, CT13, CT16, CT19, SV4, and CT32 isolates. The highest weight increment (57%) was achieved using CT32-based treatment; followed by those performed with SV4 and SV5 (55.2-55.8%) isolates (Table 3).

Bacterial isolate	Organ	Strep. and Rif. Resistance[a]	Endophytic behavior[b]	HR test[c] and hemolytic activity[d]
CT3; CT6	Root	+	+	-
CT8	Root	+	-	-
CT1; CT2; CT7; CT13; CT14; CT19; CT28; CT30; CT44; CT45; CT47; SV3; SV4; SV5; SV6; SV7; SV8	Stem	+	+	-
CT20; CT21; CT46	Stem	-	n.t	-
CT5; CT10 ; CT15; CT16; CT17; CT27; CT32; CT57; CT40; CT42; CT55; CT56	Leaf	+	+	-
CT9; CT31; CT38; CT39; CT41	Leaf	+	-	-
CT29	Leaf	-	n.t	-
CT25 ; CT43; CT51; CT49	Flower	+	+	-
CT26; CT50; CT52; CT53; CT54	Flower	+	-	-
CT48	Flower	-	n.t	-
CT36	Green fruit	+	+	-
CT24 ; CT34 ; CT35; CT37	Green fruit	+	-	-
CT4; CT23	Green fruit	-	n.t	-
CT11; CT12	Red fruit	+	+	-
CT18	Red fruit	+	-	-
CT33	Seed	+	-	-
CT22	Seed	-	n.t	-

Table 2: Bacterial isolates recovered from healthy tomato cv. Sahel plants and their endophytic behavior in tomato plants cv. Rio Grande.
[a]Bacterial colonies transferred to Nutrient Agar (NA)® (HiMedia, India) medium supplemented with 100 µg ml^{-1} (w v^{-1}) of streptomycin sulfate (Strep.) and 100 µg ml^{-1} (w v^{-1}) of rifampicin (Rif.). +: Resistance, -: Sensitivity.
[b] Endophytic progress evaluated after re-isolation, from tomato stems inoculated with isolates double resistant to streptomycin and rifampicin, on NA amended with both antibiotics 100 µg ml^{-1} (w v^{-1}), bacterial colonies similar to the wild type ones were selected and the wild type isolate was considered as endophyte and used for further bioassay. +: Presence of colonies similar to wild type ones, -: Absence of colonies and/or absence of colonies similar to the wild type ones; n.t: Not tested.
[c]Hypersensitivity (HR) test performed on tobacco plants and evaluated after 24 h of incubation at room temperature. -: Absence of chlorotic and/or necrotic zones on inoculated leaf areas.
[d]Hemolytic activity tested on Blood Agar® (HiMedia, India) medium. -: Absence of clear zone around bacterial colonies after 48 h of incubation at 25 °C.

Root development

Assessed for their ability to promote root development, CT10, SV7, CT12, CT43, SV5, CT13, CT16, CT19, SV4, and CT32 isolates led to a significant increase in the maximum root length by 19.6-28.3% compared to control. The highest increment (28.3%) was noted on plants treated with SV4 and to a lesser extent (27.7 and 27.1%) on those challenged with CT32 and SV5 (Table 3). As for the root fresh weight, CT7, CT17, CT10, CT12, CT43, SV5, CT13, CT16, CT19, SV4 and CT32 treatments significantly improved this parameter by 38.2-58.8% over control. The highest increment (58.8%) was achieved using the isolate CT32 and to a lesser extent CT19 (51.2%). For the remaining isolates, root fresh weight was also interestingly improved by 38.2-49.5% relative to control (Table 3).

Based on their effects on the four growth parameters, CT12, CT13, CT16, CT19, CT32, CT43, SV4 and SV5 were selected as being the most efficient plant growth-promoting bacterial (PGPB) isolates. The effect of some of these isolates on the aerial part growth and on the root development is illustrated in Figure 1.

Seed germination promoting ability of tomato-associated endophytic bacteria

The effect of the different bacterial treatments on the germination of tomato cv. Rio Grande seeds is shown in Figure 2. Germination of seeds bacterized with SV4 cell suspension was 40% after 2 days of incubation compared to 0% noted on control and the other treated seeds. However, after 5 days of incubation, seed germination was stimulated following CT12, CT16, CT19, CT32, CT43, SV4 and SV5 based treatments where the percentage of germination ranged between 80 and 95% compared to 60% noted on control seeds and those challenged with CT13 (Figure 2).

Characterization and identification of the most efficient PGPB isolates

The eight bacterial isolates CT12, CT13, CT16, CT19, CT32, CT43, SV4 and SV5 were morphologically and biochemically characterized and identified using 16S rDNA sequencing gene (Table 4).

Phylogenetic and Blast-N analysis of the sequenced 16S rDNA gene homology revealed that the four endophytic isolates CT32, CT43, SV4 and SV5 belonged to the genus *Bacillus* with 99.7% of similarity (for CT32) with *B. amyloliquefaciens* BD18C2-S18, 99.5% (for CT43) to *B. subtilis* subsp. *inaquosorum* 10LS6 and 99% (for SV4 and SV5, respectively) to *B. licheniformis* 262ZY2 and *B. subtilis* PDRRB2 (Table 4 and Figure 3a). The three isolates CT12, CT13 and CT16 belonged to the genus *Stenotrophomonas* with percentages of homology of about 99.7, 99.6 and 100% to *S. maltophilia* Ysm, *S. maltophilia* PPA N3 and *S. maltophilia* F70, respectively (Table 4 and Figure 3b). Blast-N analysis showed that the isolate CT19 belonged with 99.5% of similarity to *Pseudomonas geniculata* T291 and the phylogenetic tree analysis revealed a short distance between the isolate CT19 and *P. geniculata* T291 (Table 4 and Figure 3c).

The partial 16S rDNA genes of CT12, CT13, CT16, CT19, CT32, CT43, SV4 and SV5 isolates have been submitted to GenBank and have acquired the following accession numbers KR818058-KR818065, respectively (Table 4).

Plant Growth-promoting Traits Expressed in PGPB Isolates

Indole-3-acetic acid production

Assessed for their ability to produce phytohormones, the eight

Bacterial isolates	Plant height (cm)	Aerial part fresh weight (g)	Maximum root length (cm)	Root fresh weight (g)
Control	20 cd ± 0	8 fgh ± 0.1	17.2 hij ± 0.6	4.2 fgh ± 0.1
CT3	20.2 cd ± 0.6	8.4 fgh ± 0.6	20.6 abcdefghi ± 0.3	5.6 defgh ± 0.2
CT6	21.8 cd ± 0.2	8.6 fgh ± 0.2	20.8 abcdefghi ± 0.4	6.2 cdefgh ± 0.2
CT1	22 cd ± 0.8	10.2 efgh ± 0.6	20 bcdefghi ± 0.3	4 gh ± 0.3
CT2	21 cd ± 1.4	10 efgh ± 0.7	21.2 abcdefgh ± 0.6	6.4 cdefg ± 0.2
CT7	25.4 bc ± 0.6	11 efgh ± 1	19.8 cdefghi ± 0.1	6.8 bcde ± 0.2
CT13	29.8 a ± 0.9	15.6 abc ± 1	22.2 abcde ± 0.2	7.8 bc ± 0.4
CT14	22.4 cd ± 0.7	8.2 fgh ± 0.6	20.8 abcdefghi ± 0.8	6.6 bcdef ± 0.5
CT5	21 cd ± 0.7	8.8 fgh ± 0.4	18 fghij ± 0.3	4.4 efgh ± 0.2
CT10	20.2 cd ± 0.3	8.8 fgh ± 0.2	21.6 abcdef ± 0.5	7.6 bcd ± 0.2
CT15	22.4 cd ± 0.7	8.2 fgh ± 0.8	19.6 defghi ± 0.6	4 gh ± 0.3
CT16	29.8 a ± 0.4	15.2 abc ± 0.8	21.6 abcdef ± 0.3	7.6 bcd ± 0.5
CT17	22.4 cd ± 0.7	8 fgh ± 0.3	21.2 abcdefgh ± 0.6	7.8 bc ± 0.2
CT11	23.8 cd ± 1.3	10.4 efgh ± 0.3	20.6 abcdefghi ± 0.5	5.4 defgh ± 0.2
CT12	28.8 ab ± 0.6	16 abc ± 1.2	21.4 abcdefg ± 0.3	7.8 bc ± 0.2
CT19	30 a ± 0.3	16.4 abc ± 0.7	22.6 abcd ± 0.6	8.6 b ± 0.3
CT28	20 cd ± 0.7	7.2 gh ± 0.8	20.4 abcdefhi ± 0.3	5.4 defgh ± 0.7
CT27	23 cd ± 0.5	8.6 fgh ± 0.3	19.2 defghij ± 0.6	5 efgh ± 0.5
CT25	24 cd ± 0.3	9.6 efgh ± 0.3	17.4 ghij ± 0.2	6.6 bcdef ± 0.2
CT30	20 cd ± 0.7	7 h ± 0.5	15.8 j ± 0.3	3.8 h ± 0.1
CT32	31.2 a ± 1.5	18.6 a ± 0.9	23.8 ab ± 0.4	10.2 a ± 0.4
CT57	23.4 cd ± 0.2	6.8 h ± 0.2	20.4 abcdefghi ± 0.3	6.4 cdefg ± 0.2
CT44	22.4 cd ± 0.3	7.4 gh ± 0.6	20.2 abcdefghi ± 0.4	5.6 defgh ± 0.3
CT45	21.4 cd ± 0.4	7.8 fgh ± 0.7	18.4 efghij ± 0.8	4.4 efgh ± 0.2
CT47	20 cd ± 0.8	8.2 fgh ± 0.4	17.6 fghij ± 0.5	4.2 fgh ± 0.2
CT40	19.4 d ± 1	7.4 gh ± 0.4	19.2 defghij ± 0.6	5.2 efgh ± 0.3
CT42	21.2 cd ± 0.8	7.6 gh ± 0.6	20.6 abcdefghi ± 0.2	4.4 efgh ± 0.2
CT55	22.6 cd ± 0.7	7 h ± 0.3	20.4 abcdefghi ± 0.3	6.6 bcdef ± 0.3
CT56	22 cd ± 1	6.8 h ± 0.6	20.2 abcdefghi ± 0.4	6.6 bcdef ± 0.3
CT43	29.2 ab ± 0.6	16 abc ± 0.8	22.6 abcd ± 0.3	8.2 bc ± 0.4
CT49	22 cd ± 0.3	7.4 gh ± 0.9	17 ij ± 0.5	5.2 efgh ± 0.3
CT51	22.8 cd ± 0.6	7 h ± 0.3	18.4 efghij ± 0.8	6.2 cdefgh ± 0.2
CT36	19.4 d ± 0.7	7.4 gh ± 0.5	18.4 efghij ± 0.2	4.2 fgh ± 0.2
SV3	21.2 cd ± 1.3	14.53 bcd ± 0.7	19 defghij ± 1.4	5.32 efgh ± 0.1
SV4	30.2 a ± 0.8	18.12 ab ± 0.4	24 a ± 0.7	8.31 bc ± 0.4
SV5	29.2 ab ± 0.5	17.85 ab ± 0.4	23.6 abc ± 0.4	8.26 bc ± 0.2
SV6	19.6 d ± 0.8	12.13 def ± 0.1	17.4 ghij ± 0.7	5.07 efgh ± 0.2
SV7	22.6 cd ± 1.1	13.22 cde ± 0.7	21.4 abcdefg ± 1.1	5.13 efgh ± 0.2
SV8	21.4 cd ± 0.5	11.90 defg ± 0.2	19.6 defghi ± 0.3	5.15 efgh ± 0.1

Table 3: Comparative plant growth-promoting ability of endophytic bacterial isolates recovered from healthy tomato cv. Sahel on tomato cv. Rio Grande plants noted 60 days post-treatment.
Values (± standard error) sharing the same letter is not significantly different according to Student-Newman-Keuls test at P ≤ 0.05.

selected endophytic isolates (namely *S. maltophilia* CT12, *S. maltophilia* CT13, *S. maltophilia* CT16, *P. geniculata* CT19, *B. amyloliquefaciens* CT32, *B. subtilis* subsp. *inaquosorum* CT43, *B. licheniformis* SV4, and *B. subtilis* SV5) were shown able to produce the indole-3-acetic acid (IAA) after 48 h of incubation (Table 5). In fact, after 24 h of incubation, only *S. maltophilia* CT13, *S. maltophilia* CT16 and *B. amyloliquefaciens* CT32 produced IAA by 0.41, 0.79 and 2.49 µg ml^{-1}, respectively. However, after 48 h of incubation, IAA production ranged between 10.25 and 30.99 µg ml^{-1} depending on isolates. The highest IAA amount (30.99 µg ml^{-1}) was released by *B. licheniformis* SV4 (Supplementary Table 1).

Phosphate solubilization ability

S. maltophilia CT13, *S. maltophilia* CT16, *B. licheniformis* SV4 and *B. subtilis* subsp. *inaquosorum* CT43 were shown able to solubilize phosphate as indicated by the formation of a clear zone of about 10.33 to 16.83 mm in diameter around their colonies, respectively, when grown on Pikovskaya agar medium (Supplementary Table 1). However, *S. maltophilia* CT12, *P. geniculata* CT19, *B. amyloliquefaciens* CT32 and *B. subtilis* SV5 failed to produce phosphatase on this medium (Table 5).

Pectinolytic activity

The eight selected isolates were positive for pectinase activity (Table 5) which varied from 0.17-12.63 U ml^{-1}, 0.44-16.65 U ml^{-1} and 0.84-13.8 U ml^{-1} after 24, 48 and 72 h of incubation. After 24 h of incubation, the highest pectinase production (of about 12.63 U ml^{-1}) was noted in *B. subtilis* subsp. *inaquosorum* CT43. After 48 h of incubation, *S. maltophilia* CT13 exhibited the highest pectinolytic activity estimated at 16.35 U ml^{1}. After 72 h of incubation, *P. geniculata* CT19 showed the highest activity of about 13.8 U ml^{-1} (Supplementary Table 1).

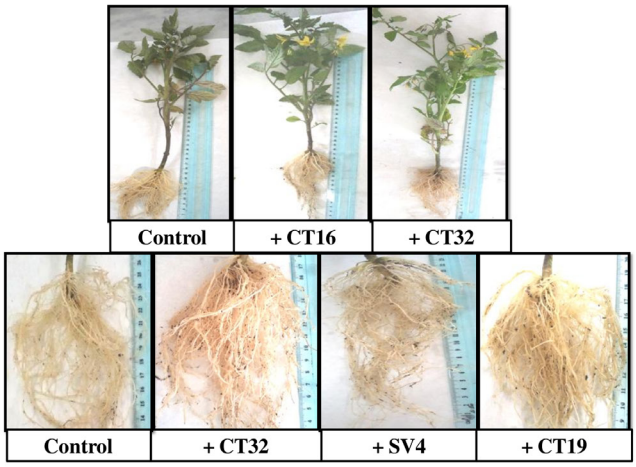

Figure 1: Effect of endophytic bacterial isolates (CT16, CT32, SV4 and CT19) recovered from healthy tomato plants on aerial part and root growth of tomato cv. Rio Grande plants noted 60 days post treatment compared to control. CT19, SV4: Isolated from stems; CT16 and CT32: Isolated from leaves.

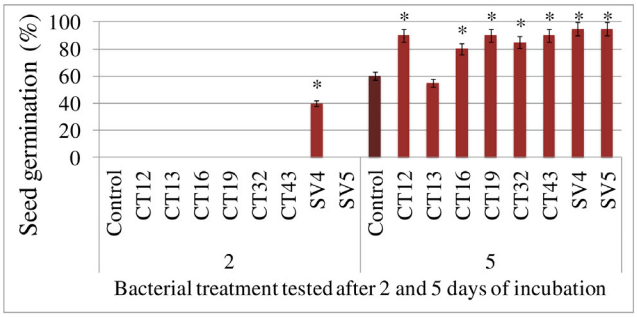

Figure 2: Effect of eight plant growth-promoting bacterial isolates recovered from healthy tomato plants on germination of tomato cv. Rio Grande seeds noted after 2 and 5 days of incubation at 25°C as compared to control. CT13, CT19, SV4 and SV5: Isolated from stems; CT16 and CT32: Isolated from leaves; CT43: Isolated from flowers; CT12: Isolated from red fruits.

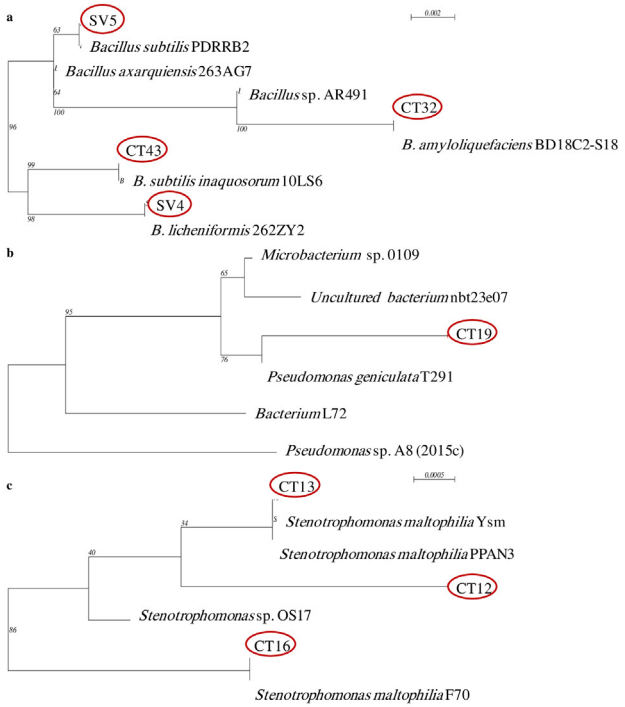

Figure 3: Neighbor-joining phylogenetic tree of partial 16S rDNA sequences of the endophytic bacterial isolates SV5, SV4, CT32, CT43, CT19, CT12, CT13 and CT16 recovered from healthy tomato plants and their closest phylogenetic relatives.

The nucleotide sequences used of representative strains were obtained from Genbank database under the following accession numbers : **a.** LN829575 (*Bacillus* sp. AR491), HE610886 (*B. amyloliquefaciens* BD18C2-S18), HQ284926 (*B subtilis* subsp. *inaquosorum* 10LS6), KF831384 (*B. licheniformis* 262ZY2), KF811050 (*B. axarquiensis* 263AG7), JN934392 (*B. subtilis* PDRRB2), **b.** KC764994 (*Pseudomonas geniculata* T291), KU221423 (Bacterium L72), DQ839620 (*Microbacterium* sp. 0109), FJ893704 (*Uncultured bacterium* nbt23e07), KT316391 (*Pseudomonas* sp. A8 (2015c), **c.** KF278963 (*Stenotrophomonas maltophilia* Ysm), JQ308603 (*S. maltophilia* PPA N3), EF491967 (*Stenotrophomonas* sp. OS17), HQ647255 (*S. maltophilia* F70), and for the bacterial isolates tested: KR818062 (CT32), KR818063 (CT43), KR818064 (SV4), KR818065 (SV5), KR818058 (CT12), KR818059 (CT13), KR818060 (CT16), KR818061 (CT19). The tree topology was constructed using ClustalX (1.81).

Discussion

This study highlight the efficiency of 38 endophytic bacterial isolates naturally associated to healthy tomato plants to promote growth of this plant. These isolates exhibited endophytic behavior when challenged to tomato cv. Rio Grande seedlings as confirmed through their re-isolation from stem tissues on PDA medium supplemented with rifampicin and streptomycin.

Tested on tomato cv. Rio Grande seedlings, eight isolates (namely CT12, CT13, CT16, CT19, CT32, CT43, SV4, and SV5) were shown able to enhance plant growth as estimated by the plant height, the aerial part and the root fresh weight and the maximum root length. In Nawangsih et al. [21] study, an endophytic *B. amyloliquefaciens* JK-SD002 isolate, recovered from tomato stems, was also shown able to improve height of treated tomato plants. Zhu et al. [33] indicated that endophytic *S. maltophilia* plays an important role in agricultural production as a plant growth-promoting bacterium. In previous studies [18,19], endophytic *Pseudomonas* spp. (*P. aeruginosa* HR7 and *Pseudomonas* sp.), recovered from healthy tomato roots and stems, also stimulated the development of this plant. Other growth parameters such as the dry weight of leaves and the number of fruits are also enhanced in tomato plants following treatments with rhizospheric and associated bacterial isolates such as *P. putida*, *P. fluorescens*, *Serratia marcescens*, *B. amyloliquefaciens*, *B. subtilis*, and *B. cereus* [34]. *P. geniculata* IC-76 recovered from nodules of cultivated chickpea showed a plant growth-promoting ability when applied either separately or in combination with five *Streptomyces* sp. isolates [35]. In our recent findings, several endophytic bacteria namely *B. cereus* S42, *Alcaligenes faecalis* S18, *B. mojavenis* S40, *S. maltophilia* S37, *Stenotrophomonas* sp. S33, *Pseudomonas* sp. S85, *B. tequilensis* SV39, *B. subtilis* SV41, *B. methylotrophicus* SV44, *Bacillus* sp. SV101, *B. tequilensis* SV104 and *B. amyloliquefaciens* subsp. *plantarum* SV65, and *Serratia* sp. C4 obtained from surface-sterilized tissues of various wild and cultivated *Solanaceae* plants were also able to enhance tomato growth in plants challenged or not with *Fusarium oxysporum* f. sp. *lycopersici* [36-40].

The eight bacterial isolates selected as being the most PGPB on tomato plants were macro-morphologically and biochemically characterized and molecularly identified by 16S rDNA gene sequencing as *S. maltophilia* CT12 (KR818058), *S. maltophilia* CT13 (KR818059), *S. maltophilia* CT16 (KR818060), *P. geniculata* CT19 (KR818061), *B. amyloliquefaciens* CT32, (KR818062), *B. subtilis* subsp.

	Bacterial isolates								
	CT12	**CT13**	**CT16**	**CT19**	**CT32**	**CT43**	**SV4**	**SV5**	
Morphological characterization									
Form	Circular	Circular	Irregular	Irregular	Irregular	Irregular	Irregular	Circular	
Margin	Regular	Regular	Curly	Lobed	Curly	Curly	Lobed	Undulate	
Elevation	Plan	Convex	Humped	Elevated	Humped	Humped	Humped	Elevated	
Surface	Smooth	Smooth	Rough	Rough	Rough	Rough	Rough	Rough	
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	
Color	Creamy	Creamy	White	White	White	White	White	White	
Gram' staining	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Positive	
Biochemical characterization									
King A	-	-	-	-	-	-	-	-	
Catalase	+	+	+	+	+	+	+	+	
Urease	+	+	-	+	-	-	-	-	
Lechitinase	-	-	+	-	+	+	-	-	
Nitrate reductase	+	+	+	+	+	+	+	+	
Tryptophane deaminase	-	-	+	-	+	+	-	-	
Lysine decarboxylase	-	-	-	-	-	-	-	-	
Mannitol	+	+	-	+	-	-	+	+	
Simmons citrate	+	+	-	+	+	-	+	+	
Indole	+	+	+	+	+	+	+	+	
Red of Methyl	-	-	+	+	+	+	-	+	
Vosges-Proskauer	+	+	-	-	-	-	+	-	
Hydrogen sulfide	-	-	-	-	-	-	-	-	
Molecular characterization (16 rDNA sequencing gene)									
Most related species	*Stenotrophomonas maltophilia* Ysm (99.7)	*Stenotrophomonas maltophilia* PPA N3 (99.6)	*Stenotrophomonas maltophilia* F70 (100)	*Pseudomonas geniculata* T291 (99.5)	*Bacillus amyloliquefaciens* BD18C2-S18 (99.7)	*Bacillus subtilis* subsp. *inaquosorum* 10LS6 (99.5)	*Bacillus licheniformis* 262ZY2 (99)	*Bacillus subtilis* PDRRB2 (99)	
Accession number	**KR818058**	**KR818059**	**KR818060**	**KR818061**	**KR818062**	**KR818063**	**KR818064**	**KR818065**	

Table 4: Characterization and identification of the plant growth-promoting bacterial isolates recovered from healthy tomato cv. Sahel plants.
Numbers in parenthesis indicate the percentage (in %) of sequence homology obtained from Blast-N analysis.

Bacterial isolates	PGP traits		
	IAA production[a]	Phosphate solubilization[b]	Pectinolytic activity[c]
Stenotrophomons maltophilia CT12 (KR818058)	+	-	+
Stenotrophomons maltophilia CT13 (KR818059)	+	+	+
Stenotrophomons maltophilia CT16 (KR818060)	+	+	+
Pseudomonas geniculata CT19 (KR818061)	+	-	+
Bacillus amyloliquefaciens CT32 (KR818062)	+	+	+
Bacillus subtilis subsp. *inaquosorum* CT43 (KR818063)	+	-	+
Bacillus licheniformis SV4 (KR818064)	+	+	+
Bacillus subtilis SV5 (KR818065)	+	-	+

Table 5: Plant growth-promoting (PGP) mechanisms of endophytic bacterial isolates recovered from healthy tomato cv. Sahel plants.
[a]IAA: Indole-3-acetic acid production after 48 h of incubation at 28 ± 2°C in Luria-Broth medium; +: Production of IAA.
[b]Tested on Pikovskaya agar medium and incubated at 28 ± 2°C for 7 days; +: Presence of clear zone; -: Absence of clear zone.
[c]Tested on NA-pectin medium (0.5 % w v^{-1}) and incubated at 28 ± 2°C for 48 h; +: Presence of clear zone.

inaquosorum CT43 (KR818063), *B. licheniformis* SV4 (KR818064), and *B. subtilis* SV5 (KR818065). Marquez-Santacruz et al. [41] found *Stenotrophomonas*, *Bacillus*, *Microbacterium*, *Pseudomonas*, and *Burkholderia* genera in the root-bacterial community of Mexican husk tomato plants with *Stenotrophomonas* being the predominant one (21.9%). Our isolates *S. maltophilia* CT12, *S. maltophilia* CT13 and *S. maltophilia* CT16 have been recovered from tomato fruits, stems and leaves, respectively. Romero et al. [2] found that *Stenotrophomonas*, *Acinetobacter* and *Pseudomonas* genera were frequently isolated from healthy tomato leaves. Munif et al. [42] also found that the most abundant endophytic bacterial genera recovered in tomato roots were *Bacillus* and *Pseudomonas*. In this study, *Bacillus* spp. isolates (*B. amyloliquefaciens* CT32, *B. subtilis* subsp. *inaquosorum* CT43, *B. licheniformis* SV4 and *B. subtilis* SV5) and *P. geniculata* CT19 were recovered from leaves, flowers and stems, respectively. Kefi et al. [43] results revealed the presence of *Bacillus* spp. (*B. mojavensis*, *B. subtilis*, *B. amyloliquefaciens*) in healthy tissues of roots, stems, leaves and fruits of tomato plants.

Bacterial isolates identified in this study improved germination of tomato cv. Rio Grande seeds by 80-95% after 5 days of incubation. Similar germination rates were recorded using two endophytic *B. subtilis* isolates, recovered from coconut and cotton, and with three rhizospheric *P. fluorescens* isolates [44]. *B. licheniformis* SV4 induced induced an earlier germination of tomato seeds, by 40%, after 2 days of incubation. However, Tabli et al. [45] demonstrated that twenty rhizospheric bacterial isolates enhanced this parameter by about 40-100% after 7 days of incubation.

As plant growth-promoting traits, the eight bacterial isolates were able to produce IAA after 48 h of incubation and interestingly *S. maltophilia* CT13, *S. maltophilia* CT16 and *B. amyloliquefaciens* CT32 produced this phytohormone earlier after 24 h of incubation. Endophytic *Pseudomonas* sp. isolates, obtained from soybean plants, showed IAA production [46]. The IAA amounts produced by our *Bacillus* spp. isolates varied from 13.37 to 30.99 µg ml^{-1} compared to 15.2-59.2 µg ml^{-1} recorded in endophytic *Bacillus* spp. (*B. megaterium*, *B. licheniformis*, *B. cereus*, *B. pumilus* and *Bacillus* sp.), obtained from healthy tomato plants and displayed an enhancement in growth parameters of treated plants [47]. Our isolates *S. maltophilia* CT16, *S. maltophilia* CT12 and *S. maltophilia* CT13 produce 10.25, 22.17 and 26.9 µg ml^{-1} of IAA, after 48 h of incubation, which were less than those produced by *S. maltophilia* TEM56 and PM22, recovered from *Amaranthus hybridus* and *Cucurbita maxima*, where IAA amounts were estimated at 0.32 and 0.49 mg ml^{-1}, respectively [48].

The phosphate solubilization ability was confirmed for *S. maltophilia* CT13, *S. maltophilia* CT16, *B. subtilis* subsp. *inaquosorum* CT43, and *B. licheniformis* SV4 siolates. In Amaresan et al. [47] study, *B. licheniformis* BESC1, recovered from healthy tomato, was unable to solubilize phosphate whereas two *Bacillus* sp. isolates were shown positive for this test. The endophytic *S. maltophilia* PM22 produced phosphatase while *S. maltophilia* TEM56 did not [48]. Phosphate solubilization ability may vary depending on isolates of a same species as is the case of *B. subtilis* CT43 in this study which was able to solubilize the phosphate whereas *B. subtilis* SV5 did not. It is also the case of *S. maltophilia* isolates where CT13 and CT16 were capable to solubilize phosphate while *S. maltophilia* CT12 did not.

The four *Bacillus* spp., the three *S. maltophilia* CT12, CT13 and CT16 and *P. geniculata* CT19 isolates, which successfully colonized tomato cv. Rio Grande stems, were shown able to produce pectinase when grown on pectin-agar medium. Pectinolytic potential may be also involved in the recorded enhancement of tomato growth as reported by Baldan et al. [49] and in the endophytic colonization of host plant [5]. It should be also mentioned that pectinases act normally as virulence factors for plant pathogenic bacteria but in case of endophytic microorganisms, they might play a role in the invasion of host plants by endophytes as demonstrated for *B. cereus*, *B. subtilis*, *B. stearothermophilus* [50] and *S. maltophilia* [51].

Conclusion

Healthy tomato plants were found to be potential sources for isolation of plant growth-promoting bacteria. The most bio-active bacteria were characterized and identified as *B. amyloliquefaciens* CT32 (KR818062), *B. subtilis* subsp. *inaquosorum* CT43 (KR818063), *B. licheniformis* SV4 (KR818064), *B. subtilis* SV5 (KR818065), *S. maltophilia* CT12 (KR818058) *S. maltophilia* CT13 (KR818059), *S. maltophilia* CT16 (KR818060) and *P. geniculata* CT19 (KR818061). The eight endophytic bacteria had stimulated the germination of tomato seeds and enhanced plant growth. Their plant growth-promoting traits and colonization ability were achieved through IAA, phosphate solubilization and pectinolytic activity.

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