

Bio-suppression of Sclerotinia Stem Rot of Tomato and Biostimulation of Plant Growth Using Tomato-associated Rhizobacteria

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Abstract

A collection of 25 rhizobacterial strains, recovered from rhizospheric soils around healthy tomato plants grown in *Rhizoctonia*-infested fields, belonging to *Bacillus amyloliquefaciens*, *B. thuringiensis*, *B. megaterium*, *B. subtilis*, *Enterobacter cloacae*, *Chryseobacterium jejuense*, and *Klebsiella pneumoniae* was screened for its suppressive effects of Sclerotinia Stem Rot of tomato caused by *Sclerotinia sclerotiorum* and plant growth-promoting ability. The inhibitory effects of diffusible and volatile metabolites from these rhizobacteria against pathogen mycelial growth depended significantly upon strains tested. Growth inhibition caused by diffusible and volatile compounds was of about 37-57% and 24-54%, respectively. All strains tested had totally suppressed myceliogenic germination of sclerotia and improved germination of bacterized tomato seeds as compared to the untreated controls. The screening of their disease-suppressive and plant growth-promoting abilities revealed 72-100% decrease in Sclerotinia Stem Rot severity and significant increments in plant height by 52-67%, roots fresh weight by about 66-88% and aerial part weight by 47-75%, compared to *S. sclerotiorum*-inoculated and untreated control. The most promising strains combining disease-suppressive and growth-promoting abilities were *B. subtilis* B10 (KT921327) and B14 (KU161090), *B. thuringiensis* B2 (KU158884), *B. amyloliquefaciens* B13 (KT951658) and B15 (KT923051), and *E. cloacae* B16 (KT921429).

Keywords: Biocontrol; Disease severity; PGPR; *Sclerotinia sclerotiorum*; Tomato

Introduction

Sclerotinia sclerotiorum (Lib.) is a serious fungus affecting yield and product quality of many susceptible hosts. It is a widespread soilborne plant pathogen with an extremely wide host range of more than 400 plant species including many of economic importance [1]. *S. sclerotiorum* is responsible for more than 60 plant diseases [2]. The pathogen produces sclerotia, which survive for long periods and attack roots of growing and mature plants, resulting in root rot, basal stem canker, and wilt [3]. Sclerotinia Stem Rot (also known as white mold or Sclerotinia Stem and Root Rot) is one of the most important tomato soil borne diseases. Plant infection occurs either by myceliogenic germination of sclerotia or by ascospores released from apothecia during carpogenic germination of sclerotia. The myceliogenically germinating sclerotia are the main source of infection on processing tomato crops leading to rotting of aerial parts of the plant in contact with soil [2,4].

Crop rotation and cultural methods are not sufficiently effective in controlling Sclerotinia Stem Rot disease because of pathogen's large host range including weeds, its ability to survive as sclerotia, and possible plant infection by airborne ascospores released from germinating sclerotia left in nearby infected fields [2,5-9]. Furthermore, no known resistance to white mold is currently available for tomato and no fungicide is currently registered for pathogen control in Tunisia. Thus, biological control using indigenous and naturally occurring microorganisms within tomato rhizosphere may be effective in controlling disease.

Biocontrol agents have received a considerable amount of attention for the control of soilborne and airborne plant diseases. Biocontrol is eco-friendly, safe and may provide long-term protection to the crop. Reduced Sclerotinia Stem Rot incidence and severity have been

demonstrated in numerous studies and successful disease control was achieved using fungi [10-14], bacteria [7,12,15-17] or biofungicides [18-20] in many cropping systems. The most efficient bacteria used for Sclerotinia Stem Rot management belonged mainly to the genera *Bacillus* [1,9,12-13,21], *Pseudomonas* [7,22], *Enterobacter* [23,24], *Serratia* [22,25-27], and at a lesser extent *Streptomyces*, *Burkholderia*, *Pantoea*, and *Paenibacillus* [22,25].

Bacteria that colonize plant roots and promote plant growth are referred to be plant growth-promoting rhizobacteria (PGPR). PGPR are highly diverse and are widely used as biocontrol agents against various plant diseases. Their disease-suppressive effects may be achieved via local antagonism toward soil borne pathogens or through induction of systemic acquired resistance against pathogens throughout the entire plant [28,29].

The objectives of the present study were to evaluate the *in vitro* antagonistic potential of 25 rhizobacterial strains, recovered from tomato rhizosphere, toward *S. sclerotiorum* growth and to assess their abilities to suppress Sclerotinia Stem Rot and to promote growth of tomato plants under greenhouse conditions.

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Materials and Methods

Plant material and growth conditions

Tomato cv. Rio Grande seedlings were used for all *in vivo* trials. Tomato seeds were surface-sterilized with 5% sodium hypochlorite for 2 min, rinsed with sterile distilled water (SDW) and air dried. They were sown in disinfected dimpled plates (230 seeds/tray 10 × 15 cm) and placed under greenhouse conditions (30 ± 4°C; 13/11 h light/dark photoperiod). Seedlings at the two-true-leaf growth stage were used and they were kept moist by watering daily until use.

Sclerotinia sclerotiorum isolation and culture

Plant pathogen used in this study is *S. sclerotiorum* isolated from tomato cvs. Kawther and Firenze plants exhibiting symptoms of stem rot collected from the experimental domain of the Regional Center of Research on Horticulture and Organic Agriculture, Chott-Mariem, Tunisia.

For pathogen isolation, single sclerotia removed from decaying stems or infected root and stem pieces were surface-sterilized by dipping in 5% sodium hypochlorite solution for 2 min, rinsed three times with SDW, dried on sterilized filter paper. These samples were then plated onto Potato Dextrose Agar (PDA) medium supplemented with streptomycin sulfate (300 mg/mL w/v) and incubated at 25°C [17].

Mycelial plugs (5 mm in diameter), taken from the edge of the actively growing colonies, were transferred to the Petri plates containing PDA to obtain pure cultures of the *S. sclerotiorum*. Stock cultures were maintained at 4°C until use [22]. The fungus was preliminarily tested for pathogenicity on tomato seedlings and re-isolated from experimentally infected ones fulfilling Koch's postulates.

Pathogen inoculum preparation

S. sclerotiorum was cultured on PDA and incubated at 25°C for 7 days before use. Ten PDA Petri plates (9 cm in diameter), covered with full mycelium growth, were macerated using a blender in 1L of SDW. The obtained mycelial suspension was used for plant inoculation [17].

Bacterial collection source and inoculum preparation

The 25 bacterial strains used in this study were isolated from the rhizospheric soils of apparently healthy and vigorous tomato plants grown in infested fields. These strains were shown able to suppress *Rhizoctonia solani* *in vitro* and *in vivo* in a previous work (Ouhaibi-Abdeljalil et al., unpublished data). They were identified using morphological, biochemical and molecular tools. They were also characterized for antibiotic producing ability (Bacillomycin D and fengycin A) and PGPR traits (IAA detection, siderophore production, phosphate solubilization). Their respective traits are summarized in Table 1.

Rhizobacterial stock cultures were maintained in Luria-Bertani broth (LB) amended with 15% glycerol and stored at -20°C. Before being used in the bioassays, stock cultures were streaked onto nutrient agar Nutrient Agar (NA) plates and incubated at 28°C for 48 h.

A loop-ful of these cultures was transferred to 10 ml of SDW and thoroughly shaken. One ml of this bacterial suspension was added to 300 ml of LB broth (containing 1% peptone, 0.5% yeast extract and 1% NaCl) and they were incubated on a rotary shaker at 170 rpm for 48 h. This liquid culture was diluted into 1L and the concentration of the whole cell suspension was adjusted to approximately 10⁸ cells ml⁻¹

before being used for plant treatment through culture substrate drenching [1].

In vitro screening of the antagonistic potential of bacterial strains

The antagonistic potential of the 25 rhizobacterial strains against *S. sclerotiorum* was assessed *in vitro* using the dual culture and the sealed plate techniques for bioactive diffusible and volatile compounds, respectively.

Molecular identification		Detection of antibiotic biosynthesis genes		PGPR abilities		
Strains	Accession No.	Fen A	Bac D	IAA	P. Solubilization	Siderophore production
<i>Bacillus megaterium</i> B1	KU168423	-	+	+	-	++
<i>B. thuriengensis</i> B2	KU158884	-	+	+	+	++
<i>Enterobacter cloacae</i> B3	KT923049	+	-	+	+	+++
<i>E. cloacae</i> B4	KT923050	+	+	+	+	+++
<i>B. megaterium</i> B5	KT923054	+	-	+	+	++
<i>B. subtilis</i> B6	KT921427	+	+	-	+	-
<i>B. amyloliquefaciens</i> B7	KT921428	+	+	+	-	+++
<i>B. subtilis</i> B8	KU158885	+	+	+	+	+
<i>B. amyloliquefaciens</i> B9	KU158887	+	+	+	+	+
<i>B. subtilis</i> B10	KT921327	+	+	+	+	+++
<i>Chryseobacterium jejuense</i> B11	KU158886	-	+	+	+	++
<i>Klebsiella pneumoniae</i> B12	KT921328	+	-	+	+	+++
<i>B. amyloliquefaciens</i> B13	KT951658	+	-	+	+	++
<i>B. subtilis</i> B14	KU161090	+	-	-	-	+++
<i>B. amyloliquefaciens</i> B15	KT923051	-	+	-	-	+
<i>E. cloacae</i> B16	KT921429	+	+	+	+	+++
<i>B. subtilis</i> B17	KT923055	+	+	+	+	-
<i>B. amyloliquefaciens</i> B18	KT923052	+	+	+	+	-
<i>B. subtilis</i> B19	KT921430	+	-	-	-	+++
<i>B. subtilis</i> B20	KT921431	+	+	+	+	+++
<i>B. amyloliquefaciens</i> B21	KT923047	+	+	+	+	-
<i>B. amyloliquefaciens</i> B22	KT923053	+	+	+	+	-
<i>B. thuriengensis</i> B23	KT923056	+	+	+	+	+
<i>B. megaterium</i> B24	KT923048	-	+	-	-	+++
<i>B. subtilis</i> B25	KU161091	-	+	-	-	+

Table 1: Tomato-associated rhizobacterial strains used and their main traits. Fen A: Fengycin A; Bac: Bacillomycin, IAA: Indole acetic acid; P.solubilization: Phosphate solubilization. All isolates were negative for hypersensitive reaction (HR) on tobacco leaves, and they were negative for the detection of Fengycin B, D and E. Positive reaction (+); Negative reaction (-); Production of siderophore was scored as non-detected (-), low (+), middle (++) and high (+++).

Assessment of the antifungal activity of diffusible compounds

S. sclerotiorum cultures were grown on PDA at 25°C during 5 days. Agar plugs (5 mm in diameter), cut from actively growing cultures, were placed on the extremity of a Petri plate (9 cm in diameter) containing PDA. For each bacterial strain, 10 µl of cell suspension (10⁸ cells ml⁻¹), prepared from 48 h-old culture, was dropped in a well (5 mm in diameter) made at the opposite edge of the plate. Plates incubated with fungal agar plugs alone were used as control. Plates were incubated at 25°C for 5 days. Each individual treatment was replicated thrice. Pathogen colony diameter and the inhibition zone were measured and compared with the untreated control. The percentage of inhibition of *S. sclerotiorum* radial growth was calculated using the following formula [30]:

$$GI (\%) = \frac{(R - r) \times 100}{R}$$

Where GI the percent of inhibition in growth of test pathogen, R is the colony diameter of the pathogen in the control plate and r is the colony diameter in treated plate.

Assessment of the antifungal activity of volatile compounds

The antagonism due to volatile compounds of the 25 strains tested was evaluated using the sealed plate method [31] with some modifications. For this test, 10 µl of 48 h-old rhizobacterial cell suspension adjusted to (10⁸ cells ml⁻¹) were dropped into wells (5 mm in diameter) in Petri plates (90 mm in diameter) containing NA. A 5 mm agar plug removed from a 5-day-old *S. sclerotiorum* culture was placed in the centre of a second Petri plate containing PDA, then the fungal plate was inverted over the bacterial plate. Both half plates were wrapped with parafilm to seal in the bacterial volatile compounds. The plates were incubated at 25°C for 5 days. Control set of paired plates was designed with only the test fungus on PDA half plate inverted over untreated nutrient agar half plate. The experiment was conducted in triplicate. After the incubation period, the paired plates were observed for mycelial growth inhibition as compared to the untreated control. Percentage inhibition of the mycelial growth of the fungus was calculated as mentioned above in the dual culture method [30].

Effect of bacterial strains on sclerotia germination

The antagonistic potential of the 25 rhizobacterial strains was also tested on sclerotial germination of *S. sclerotiorum* according to the procedure of Zizzerini et al. [32] with slight modifications. Ten-day-old sclerotia of the pathogen formed on PDA were collected and surface-sterilized with 2% sodium hypochlorite solution, and rinsed three times with SDW. Rhizobacteria cell suspensions, prepared as described above, were grown in Erlenmeyer flasks containing 10 ml of LB medium. Five sclerotia were suspended in each bacterial liquid culture and incubated for 24 h on a rotary shaker at 175 rpm. Control sclerotia were suspended in Erlenmeyer flasks containing LB medium only. The bacterized sclerotia were gently removed and placed onto PDA plates (3 sclerotia per each plate) and subsequently incubated for 5 days at 25°C. Germination of sclerotia was noted and compared to untreated control ones.

Effect of bacterial strains on seed germination

The bacterial strains were screened for their ability to enhance germination of tomato cv. Rio Grande seeds. Seeds were sterilized by dipping for 2 min in 5% sodium hypochlorite solution then rinsed three times with SDW. Bacterial cell suspensions were prepared as described above and a volume of 1 ml was individually added to Erlenmeyer flasks

containing 20 ml of LB medium and 12 tomato seeds were suspended in those suspensions. Seeds placed in LB medium uninoculated with rhizobacterial strains were used as control. Then, Erlenmeyer flasks were incubated on a rotary shaker at 150 rpm for 24 h. Seeds were recuperated, dried on a sterile filter paper and placed in Petri plate containing two layers of sterile filter paper moistened with SDW and incubated in the dark at 28°C for one week. SDW was added when needed to the filter papers to keep them moist. Seeds were considered as germinated and counted when the radicle protruded through the seed coat [33]. The percentage of germination was calculated according to the following formula:

% G = (NGS/12) × 100 where NGS is the number of germinated seeds.

Assessment of Sclerotinia Stem Rot-suppressive and plant growth-promoting abilities

The antagonistic potential of the 25 rhizobacterial strains tested against *S. sclerotiorum* was assessed under greenhouse conditions for disease suppression and plant growth promotion abilities. The trial was conducted at the experimental domain of the Regional Center of Research on Horticulture and Organic Agriculture in Chott-Mariem, Tunisia. Rhizobacteria and pathogen cultures were prepared as described above. Inoculation was performed on 21-day-old tomato cv. Rio Grande seedlings. In each socket containing a tomato plant, 30 ml of a bacterial cell suspension (adjusted to 10⁸ cells ml⁻¹) was drenched at the collar level. One week after bacterial treatment, 30 ml of the fungal inoculum were poured at the same level to each plant. The untreated controls were watered with SDW only. One day after pathogen challenge, the plants were transferred into pots (16 cm in diameter) prepared 7 days before transplanting where each pot containing peat was watered with 40 ml of fungal inoculum. A reminder treatment by the bacterial suspension was made 24 h after transplanting [34].

The following treatments were included in the experiment:

- Uninoculated and untreated tomato plants with neither pathogen nor bacteria (positive control)
- Tomato plants inoculated with *S. sclerotiorum* only
- Tomato plants inoculated with *S. sclerotiorum* and treated with each of the rhizobacterial strains tested.

Two months after inoculation and treatment, the plant height and the aerial part and root fresh weights were recorded. Disease severity on collar and roots was also assessed using an arbitrary 0-5 scale where: 0=no symptom, 1=0-25% of root browning, 2=26-50% of root browning, 3=51-75% of root browning, 4=76-100% of root browning, and 5=plant death. Disease incidence was also estimated using the following formula:

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected plants} \times 100}{\text{Total number of plants}}$$

Statistical analysis

The results were subjected to one-way analysis of variance and means separation was performed using the Duncan's Multiple Range test at ($P \leq 0.05$). ANOVA was carried out using SPSS version 16.0. The tests were conducted according to a completely randomized design both for the *in vitro* (screening of the antagonistic potential of the bacterial isolates, 3 replications) and the *in vivo* (disease index and plant growth parameters, 5 replications) bioassays. For the *in vivo* trials, 27 individual treatments were tested corresponding to 25

bacteria-based treatments, an uninoculated and untreated control and a *S. sclerotiorum*-inoculated and untreated control. The relationships between Sclerotinia Root Rot index and plant growth parameters were compared using Pearson's correlation analysis at $P \leq 0.05$.

Results

Growth-suppressing effects of diffusible metabolites from tomato-associated rhizobacteria toward *S. sclerotiorum*

ANOVA analysis revealed that pathogen radial growth, noted after 5 days of incubation at 25°C, varied significantly (at $P \leq 0.05$) depending on antagonistic treatments tested. In fact, data from the dual culture assay given in Table 2 showed that all the isolates had significantly (at $P \leq 0.05$) inhibited the mycelial growth of *S. sclerotiorum*. The percentage of growth reduction, as compared to untreated control, varied between 37.22 and 56.67% and exceeded 40% for 23 out of 25 strains tested.

It should be highlighted that more than 50% decrease in *S. sclerotiorum* radial growth was achieved using *B. thuringiensis* B2 (KU158884) and B23 (KT923056), *B. subtilis* B19 (KT921430), *B. amyloliquefaciens* B22 (KT923053) and B9 (KU158887), and *E. cloacae* B16 (KT921429) strains.

The results of this dual culture assay also indicated that the tested rhizobacteria led to formation of antibiosis zones when confronted with *S. sclerotiorum* (Figure 1). The diameters of the inhibition zones ranged between 0 and 13 mm depending on antagonistic treatments and exceeded 5 mm for 16 out of the 25 strains tested (Table 2). Diffusible

metabolites from *E. cloacae* B16 (KT921429), *B. amyloliquefaciens* B7 (KT921428) and B9 (KU158887), *B. thuringiensis* B2 (KU158884), *B. subtilis* B10 (KT921327), and *B. megaterium* B1 (KU168423) strains resulted in the formation of inhibition zones exceeding 11 mm in diameter.

Growth-suppressing effects of volatile metabolites from tomato-associated rhizobacteria toward *S. sclerotiorum*

ANOVA results showed that the colony diameter of *S. sclerotiorum* varied significantly ($P \leq 0.05$) depending on antagonistic treatments tested. In fact, data given in Table 2 revealed that *S. sclerotiorum* growth decrease, due to the inhibitory effects of volatile metabolites from the rhizobacterial strains tested, varied from 24.07 to 54.44% and exceeded 30% using 21 out of the 25 strains tested. Volatile compounds from *B. subtilis* B10 (KT921327), *E. cloacae* B16 (KT921429), *B. thuringiensis* B2 (KU158884), *B. megaterium* B1 (KU168423), *B. subtilis* B6 (KT921427) and B19 (KT921430), and *C. jejuense* B11 (KU158886) were the most bioactive against *S. sclerotiorum* leading to 40-54% decrease in pathogen radial growth as compared to the untreated control. Also, a delay in the formation of sclerotia, as compared to untreated control cultures, was induced by volatile metabolites from tomato-associated bacterial strains as shown in Figure 1 using *B. thuringiensis* B2 (KU158884), *B. subtilis* B10 (KT921327), and *E. cloacae* B16 (KT921429) strains.

Sclerotial germination-suppressive activities of tomato-associated rhizobacteria

Sclerotia of *S. sclerotiorum* previously exposed for 24 h to

Antagonistic treatment	Strain	Diffusible metabolites			Volatile metabolites	
		Colony diameter (mm) ¹	Growth Inhibition (%) ²	Inhibition zone (mm) ¹	Colony diameter (mm) ¹	Growth Inhibition (%) ²
<i>Bacillus megaterium</i>	B1	51.17 bcde	43.14	11.67 abc	52.5 def	41.67
<i>B. thuringiensis</i>	B2	39 f	56.67	12 ab	48.67 efg	45.92
<i>Enterobacter cloacae</i>	B3	46.83 bcdef	47.97	0 f	54.67 cde	39.26
<i>E. cloacae</i>	B4	45.5 cdef	49.44	5.33 cdef	59.17 bcde	34.26
<i>B. megaterium</i>	B5	52.33 bc	41.86	4 ef	65.33 bc	27.40
<i>B. subtilis</i>	B6	49.83 bcde	44.63	3.67 ef	53.67 cde	40.37
<i>B. amyloliquefaciens</i>	B7	48.17 bcdef	46.48	11.33 abcd	60.0 bcde	33.33
<i>B. subtilis</i>	B8	50.67bcde	43.7	2.67 ef	57.67 bcde	35.93
<i>B. amyloliquefaciens</i>	B9	44.67 cdef	50.37	12.67 a	56.83 bcde	36.85
<i>B. subtilis</i>	B10	46.33 bcdef	48.52	12 ab	41.0 g	54.44
<i>Chryseobacterium jejuense</i>	B11	45.33 cdef	49.63	5.3 cdef	53.3 cde	40.74
<i>Klebsiella pneumoniae</i>	B12	49.17 bcdef	45.37	4 ef	63.83 bcd	29.07
<i>B. amyloliquefaciens</i>	B13	46.83 bcdef	47.97	1.33 f	61.67 bcd	31.48
<i>B. subtilis</i>	B14	49.5 bcdef	45	8 abcde	56.5 bcde	37.22
<i>B. amyloliquefaciens</i>	B15	46.5 bcdef	48.33	5 def	55.33 cde	38.52
<i>E. cloacae</i>	B16	41.67 cdef	53.7	13 a	42.0 fg	53.33
<i>B. subtilis</i>	B17	45.17 cdef	49.81	6 bcdef	57.17 bcde	36.48
<i>B. amyloliquefaciens</i>	B18	46.17 bcdef	48.7	5.67 bcdef	63.0 bcd	30.0
<i>B. subtilis</i>	B19	40.67 ef	54.81	4.67 ef	53.33 cde	40.74
<i>B. subtilis</i>	B20	56.33 b	37.41	5.33 cdef	62.5 bcd	30.56
<i>B. amyloliquefaciens</i>	B21	49 bcdef	45.55	6 bcdef	68.33 b	24.07
<i>B. amyloliquefaciens</i>	B22	41 def	54.44	8.33 abcde	63.5 bcd	29.44
<i>B. thuringiensis</i>	B23	42.67 cdef	52.59	3.33 ef	61.67 bcd	31.48
<i>B. megaterium</i>	B24	56.5 b	37.22	4.33 ef	61.17 bcd	32.04
<i>B. subtilis</i>	B25	51.67 bcd	42.59	6 bcdef	58.17 bcde	35.37
Untreated control		90 a	0	0 f	90 a	0

Table 2: Growth-suppressing effects of diffusible and volatile metabolites from tomato-associated rhizobacteria toward *Sclerotinia sclerotiorum* noted after 5 days of incubation at 25°C.

¹For each parameter, values followed by the same letter are not significantly different according to Duncan's Multiple Range test (at $P \leq 0.05$).

²Percentage of growth inhibition calculated using Rostami et al. [30] formula.

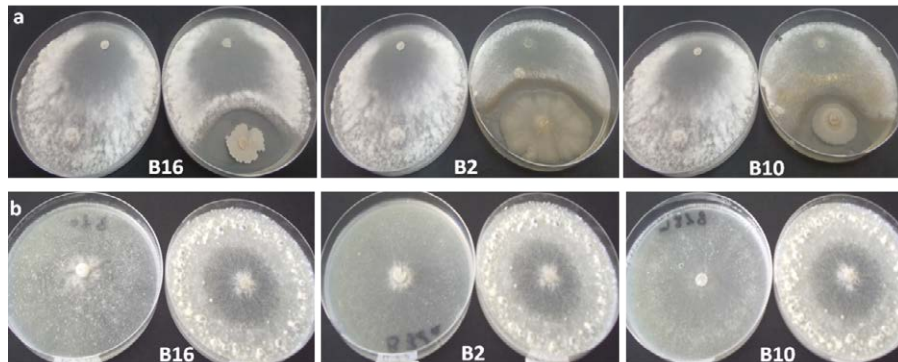


Figure 1: *In vitro* growth inhibition of *Sclerotinia sclerotiorum* due to diffusible (a) and volatile (b) metabolites from tomato-associated rhizobacteria strains compared to the untreated control. B16: *Enterobacter cloacae* B16 (KT921429); B2: *Bacillus thuringiensis* B2 (KU158884); B10: *B. subtilis* B10 (KT921327); a: Dual culture method (control plate on the left); b: Sealed plate method (control plate in the right).

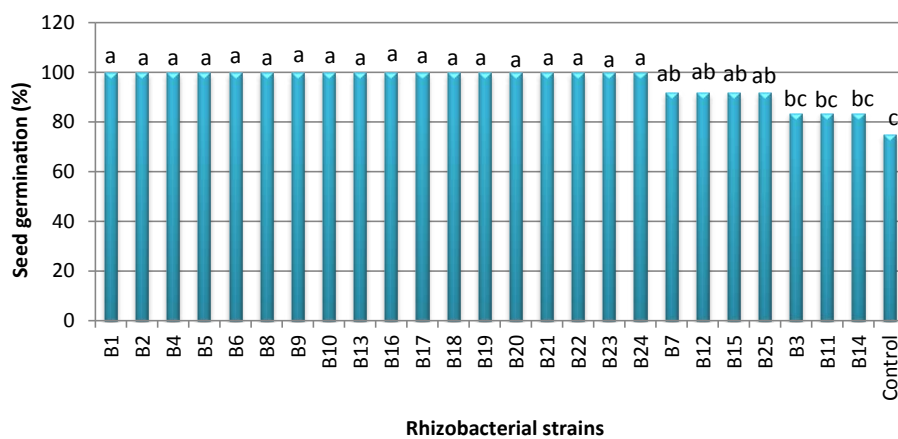


Figure 2: Effects of different rhizobacteria strains tested on germination of tomato cv. Rio Grande seeds. Each value represents the mean of three replicates and bars sharing the same letter are not significantly different according to Duncan's Multiple Range test (at $P \leq 0.05$). B1-B25: Rhizobacteria strains recovered from rhizospheric soils around healthy tomato plants.

rhizobacterial liquid cultures failed to germinate on PDA even after 5 days of incubation at 25°C. These results indicated that all strains tested had totally suppressed myceliogenic sclerotial germination as compared to the untreated controls which exhibited 100% germination. This loss of viability was expressed by the absence of growing mycelium emerging from incubated sclerotia that failed to grow even when replated on fresh PDA plates. Also, treated sclerotia showed impaired consistency. This germination suppression was associated with bacterial growth developing around treated sclerotia.

Seed germination-improving effects of tomato-associated rhizobacteria

Tomato cv. Rio Grande seeds' germination percentage noted 7 days post-treatment, varied significantly (at $P \leq 0.05$) depending on antagonistic treatments tested. In fact, data given in Figure 2 revealed that for 22 out of the 25 strains tested, seed germination potential was significantly increased compared to the untreated control. Percentage germination of bacterized tomato seeds ranged between 83.33 and 100% in contrast to 75% noted on the untreated control ones.

Suppression of Sclerotinia Stem Rot using tomato-associated rhizobacteria

Sclerotinia Stem Rot incidence, noted two months post-planting

and estimated based on the presence of root rotting signs whatever the disease index recorded, varied from 0 to 100% depending on antagonistic treatments tested (Table 3). The highest disease incidence (100%) was noted on tomato plants inoculated with *S. sclerotiorum* and treated with *K. pneumoniae* B12 (KT921328) and *C. jejuense* B11 (KU158886) strains which was comparable to that of the inoculated and untreated control. It should be also highlighted that plant treatments using *B. thuringiensis* B2 (KU158884), *B. subtilis* B10 (KT921327), *B. amyloliquefaciens* B13 (KT951658), *B. amyloliquefaciens* B15 (KT923051), and *E. cloacae* B16 (KT921429) led to total suppression of disease development and using 8 out of the 25 strains tested, disease incidence did not exceed 20% as compared to 100% recorded on pathogen-inoculated and untreated control. Sclerotinia Stem Rot severity, noted two months post-planting, depended significantly (at $P \leq 0.05$) upon the tested antagonistic treatments. As given in Table 3, this parameter ranged from 0 to 1.2 (on a 0-5 scale) for all the rhizobacteria-based treatments and disease severity scores were significantly ($P \leq 0.05$) lower than that noted on *S. sclerotiorum*-inoculated and untreated control plants (disease index 4.4).

It should be underlined that disease index records were less than 1 and significantly similar to that of the uninoculated and untreated controls for plants treated with 22 out of the 25 strains tested. This indicated that total suppression of disease development was achieved

Antagonistic treatment tested	Strain	Disease Incidence (%)	Disease index ¹	Root fresh weight (g) ²	Aerial parts' fresh weight (g) ²	Plant height (cm) ²
<i>Bacillus megaterium</i>	B1	20	0.25 cd (94.32)	8.12 defg (81.65)	14.57 fg (47.57)	47 hi (53.19)
<i>B. thuringiensis</i>	B2	0	0.0 d (100.0)	12.22 a (87.80)	30.01 a (74.54)	67 a (67.16)
<i>Enterobacter cloacae</i>	B3	60	0.6 bcd (86.36)	9.86 bc (84.89)	24.69 bc (69.06)	57 cdef (61.40)
<i>B. subtilis</i>	B4	80	1.2 b (72.73)	7.97 cdef (81.30)	16.15 efg (52.69)	51 efghi (56.86)
<i>B. megaterium</i>	B5	20	0.2 cd (95.45)	7.39 defg (79.83)	16.00 efg (52.25)	46 hi (52.17)
<i>E. cloacae</i>	B6	60	0.6 bcd (86.36)	8.77 cde (83.01)	17.71 defg (56.86)	50 fghi (56.0)
<i>B. amyloliquefaciens</i>	B7	40	0.4 cd (90.91)	7.36 defg (79.76)	18.15 defg (57.91)	60 abcd (63.33)
<i>B. subtilis</i>	B8	20	0.4 cd (90.91)	7.20 defg (79.31)	19.15 def (60.10)	57 cdef (61.40)
<i>B. amyloliquefaciens</i>	B9	20	0.4 cd (90.91)	10.01 abc (85.11)	21.24 cd (64.03)	56 cdefg (60.71)
<i>B. subtilis</i>	B10	0	0.0 d (100.0)	11.44 ab (86.97)	30.71 a (75.12)	66 ab (66.67)
<i>Chryseobacterium jejuense</i>	B11	100	1.0 bc (77.27)	9.21 cd (83.82)	17.65 defg (56.71)	53 cdefghi (58.49)
<i>Klebsiella pneumoniae</i>	B12	100	1.0 bc (77.27)	7.53 defg (80.21)	16.03 efg (52.34)	51 efghi (58.86)
<i>B. amyloliquefaciens</i>	B13	0	0.0 d (100.0)	8.79 cde (83.05)	24.28 bc (68.53)	52 defghi (57.69)
<i>B. subtilis</i>	B14	20	0.2 cd (95.45)	11.44 ab (86.97)	26.88 ab (71.58)	59 bcde (62.71)
<i>B. amyloliquefaciens</i>	B15	0	0.0 d (100.0)	11.62 ab (87.17)	24.13 bc (68.34)	61abc (63.93)
<i>E. cloacae</i>	B16	0	0.0 d (100.0)	11.62 ab (87.17)	28.50 a (73.19)	66 ab (66.67)
<i>B. subtilis</i>	B17	40	0.4cd (90.91)	6.75 efgh (77.92)	19.46 def (60.74)	48 ghi (54.17)
<i>B. amyloliquefaciens</i>	B18	40	0.4 cd (90.91)	5.83 fgh (74.44)	17.66 defg (56.74)	52 defghi (57.69)
<i>B. subtilis</i>	B19	60	0.6 bcd (86.36)	7.18 defg (79.28)	17.42 defg (56.14)	51 efghi (56.86)
<i>B. subtilis</i>	B20	20	0.4 cd (90.91)	6.20 fgh (75.96)	17.8 defg (57.07)	54 cdefgh (59.26)
<i>B. amyloliquefaciens</i>	B21	60	0.6 bcd (86.36)	4.78 hi (68.83)	18.30 def (58.25)	48 ghi (54.17)
<i>B. amyloliquefaciens</i>	B22	40	0.6 bcd (86.36)	7.18 fgh (79.25)	17.42 def (56.14)	51 i (56.86)
<i>B. thuringiensis</i>	B23	20	0.2cd (95.45)	6.57 efgh (77.32)	20.19 de (62.16)	49 fghi (55.10)
<i>B. megaterium</i>	B24	20	0.2 cd (95.45)	5.55 gh (73.15)	17.54 defg (56.44)	50 fghi (56.0)
<i>B. subtilis</i>	B25	60	0.6 bcd (86.36)	4.50 hi (66.89)	17.08 defg (55.27)	47 hi (53.19)
Untreated control	C	20	0.2 cd (95.45)	2.72 ij (45.22)	13.96 g (45.27)	32 j (31.25)
<i>S. sclerotiorum</i> -inoculated control	P	100	4.4 a (0.0)	1.49 j (0.0)	7.64 h (0.0)	22 k (0.0)

Table 3: Sclerotinia Stem Rot-suppressive and plant growth-promoting effects of tomato-associated rhizobacteria noted on tomato plants cv. Rio Grande 60 days post-planting. Sclerotinia Root Rot severity was assessed using an arbitrary 0-5 scale where: 0=no symptom and 5= 100% of root browning.

1: Values in parenthesis indicate the percentage (in %) of decrease in disease severity as compared to *S. sclerotiorum*-inoculated and untreated control plants.
 2: Values in parenthesis indicate the percentage (in %) of increase in plant growth parameters as compared to *S. sclerotiorum*-inoculated and untreated control plants.
 For each parameter, values followed by the same letter are not significantly different according to Duncan's Multiple Range test (at $P \leq 0.05$).

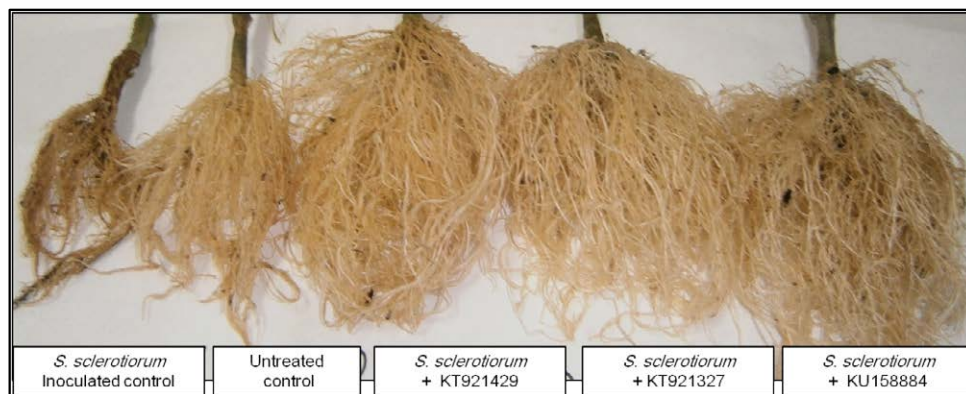


Figure 3: Sclerotinia Stem Rot severity and increased root growth achieved using tomato-associated rhizobacteria strains compared to the untreated and *S. sclerotiorum*-inoculated control. KU158884: *Bacillus thuringiensis* B2; KT921327: *B. subtilis* B10; KT921429: *Enterobacter cloacae* B16.

using these tomato-associated rhizobacterial strains where stem rot severity was reduced by 86-100% compared to *S. sclerotiorum*-inoculated and untreated control (Table 3 and Figure 3).

B. thuringiensis B2 (KU158884), *B. subtilis* B10 (KT921327), *B. amyloliquefaciens* B13 (KT951658) and B15 (KT923051), and *E. cloacae* B16 (KT921429) had totally suppressed Sclerotinia Root Rot incidence and severity. Moreover, 90-95.45% decrease in disease severity was

achieved using *B. megaterium* (B1 (KU168423), B5 (KT923054), and B24 (KT923048)), *B. amyloliquefaciens* (B7 (KT921428), B9 (KU158887) and B18 (KT923052)), *B. subtilis* (B8 (KU158885) B14 (KU161090), B17 (KT923055) and B20 (KT921431)), and *B. thuringiensis* B23 (KT923056) strains. Treatments with *K. pneumoniae* B12 (KT921328), *C. jejuense* B11 (KU158886), and *E. cloacae* B3 (KT923049) strains led to 72-86% lower disease severity compared to the inoculated and untreated control (Table 3).

Tomato growth enhancement using tomato-associated rhizobacteria

The 25 rhizobacterial strains were also evaluated for their plant growth-promoting abilities based on various growth parameters compared to the untreated control plants (*S. sclerotiorum*-inoculated or not). ANOVA results revealed that the plant height and the aerial part and roots fresh weights depended significantly ($P \leq 0.05$) upon antagonistic treatments tested. Their relative effects on each parameter were quantified below.

Plant height promotion

All the rhizobacterial strains tested had significantly (at $P \leq 0.05$) increased the plant height of the *S. sclerotiorum*-inoculated and treated plants as compared to the inoculated and untreated ones (Table 3). This increase, as compared to pathogen-inoculated control, ranged from 52.17 to 67.16% and exceeded 60% using 9 out of the 25 strains tested.

The highest plant height increment, of about 63-67% compared to the inoculated control and untreated control, were recorded on tomato plants treated with *B. thuringiensis* B2 (KU158884), *B. subtilis* B10 (KT921327), *E. cloacae* B16 (KT921429), *B. amyloliquefaciens* B7 (KT921428) and B15 (KT923051) strains. Moreover, plant treatments using these five isolates also led to significant plant height increment by 47-52% compared to healthy (disease-free) and untreated control plants (Table 3).

Aerial parts' fresh weight promotion

Data given in Table 3 revealed that all the rhizobacterial strains tested had significantly (at $P \leq 0.05$) increased the aerial parts' fresh weight as compared to *S. sclerotiorum*-inoculated and untreated control. This parameter increment ranged between 47.57 and 74.54% depending on bacterial treatments tested and exceeded 60% using 11 out of the 25 strains tested.

Based on their ability to enhance the aerial parts' fresh weight of tomato plants already challenged with *S. sclerotiorum*, bacterial treatments based on *B. thuringiensis* B2 (KU158884), *B. subtilis* B10 (KT921327), *E. cloacae* B16 (KT921429), and *B. subtilis* B14 (KU161090) strains were found to be the most effective in increasing plant aerial parts' growth by more than 71% compared to the inoculated and untreated control. This growth-promoting effect, by 48-54%, was significantly higher than that noted on the uninoculated and untreated (i.e. disease free and untreated) control plants.

Root fresh weight promotion

Results illustrated in Table 3 indicated that all rhizobacteria-based treatments applied to pathogen-inoculated plants had significantly (at $P \leq 0.05$) enhanced the roots fresh weight compared to the inoculated and untreated control ones. In fact, root fresh weight increase, compared to *S. sclerotiorum*-inoculated and untreated control plants, ranged from 66.89 to 87.80% depending on rhizobacterial strains tested and reached up to 73% using 23 out of the 25 strains tested and exceeded 80% using 13 strains.

The greatest root growth-promoting effects, expressed by more than 85% increase in root fresh weight, were achieved using *B. thuringiensis* B2 (KU158884), *E. cloacae* B16 (KT921429), *B. amyloliquefaciens* B15 (KT923051) and B9 (KU158887), and *B. subtilis* B10 (KT921327) and B14 (KU161090) strains. Furthermore, plant treatments using these six strains led to significant improvement of root growth by 72-78% when compared to the uninoculated and untreated control plants. It should be also highlighted that root fresh weight increase compared to the

uninoculated and untreated control, noted on plants already inoculated with the pathogen and achieved using the 25 strains, ranged between 39 and 78%. This indicates that these tomato-associated rhizobacterial strains have additionally bio-fertilizer properties.

Correlation between Sclerotinia Stem Rot severity and plant growth parameters

Pearson's correlation analysis revealed that plant height was significantly and negatively related to disease index ($r = -0.555$; $P = 2.7046$ E-12) indicating that increased Sclerotinia Stem Rot severity led to plant stunting. Similar trend was noted between the aerial parts' fresh weight and disease severity scores where a significant and negative correlation was detected between both variables ($r = -0.482$; $P = 3.0569$ E-9). Also, the root fresh weight was found to be negatively related to Sclerotinia Stem Rot index ($r = -0.4338$; $P = 1.4718$ E-7).

This analysis indicated that the reduced Sclerotinia Stem Rot severity recorded on tomato plants, achieved using rhizobacteria-based treatments, was related and associated to the registered growth promotion.

Discussion

Fungal soilborne diseases are among the most serious problems threatening tomato cropping in Tunisia due to their difficult control attributed mainly to the long survival of pathogens' resting structures, their wide host range and lack of genetic resistance. Furthermore, excessive application of chemical fertilizers and pesticides has led to health and environmental problems. Thus, searching for alternative control strategies that can ensure competitive yields while protecting human, plant and soil health are increasingly required [35].

The widely studied biocontrol agents for the management of *S. sclerotiorum* are mycoparasites such as *Coniothyrium minitans* and *Sporidesmium sclerotivorum* [36]. However, few attempts have been made to explore the potential use of bacterial biocontrol agents for the management of *Sclerotinia* diseases [12,15,17]. In the present study, 25 bacterial strains recovered from rhizospheric soils around healthy tomato plants and belonging to *Bacillus*, *Chryseobacterium*, *Enterobacter*, and *Klebsiella* genera were assessed for their ability to suppress Sclerotinia Stem Rot disease and to promote tomato growth. Species represented in the recovered collection of tomato-associated rhizobacteria were *B. subtilis*, *B. amyloliquefaciens*, *B. megaterium*, *B. thuringiensis*, *E. cloacae*, *C. jejuense*, and *K. pneumoniae*.

The *in vitro* trials through dual antagonist-pathogen cultures could potentially indicate the potential of some microorganisms to produce antifungal chemicals or to act as biocontrol agents [37]. In the current study, all rhizobacterial strains tested had significantly inhibited pathogen growth and the most efficient ones belong to *B. subtilis*, *B. amyloliquefaciens*, *B. thuringiensis*, *B. megaterium* and *E. cloacae* and at a lesser extent, *C. jejuense*. Their antifungal effects involved the formation of antibiosis zones due to diffusible metabolites and production of volatile compounds. These strains were shown to be Fengycin A and/or Bacillomycin D producers (Table 1). Microorganisms acting through antibiosis, generally have a wide action spectrum, and thus pathogen inhibition by producing toxic substances is more effective than any other mechanism of action [38]. These cyclic lipopeptide antibiotics have been reported to inhibit various phytopathogenic fungi including *S. sclerotiorum* [39,40]. In fact, members of *Bacillus* genus are among the beneficial bacteria mostly exploited as biopesticides [41]. Their protective effect involves different mechanisms of action that directly antagonize pathogen growth. *B.*

subtilis group is known to produce a variety of bioactive metabolites leading to antibiosis [42,43] and able to compete for space and nutrients [40]. Lipopeptides' production by *B. subtilis* play a major role in the successful control of damping-off tomato [44]. The bacterial strains are also able to inhibit the growth of sclerotium-forming phytopathogenic fungi by an antibiosis mechanism through the release of protease-resistant and thermo-stable compounds diffusible into the culture medium [45]. Similarly, Zhang et al. [46] working with *B. subtilis* also note inhibition zones of 10-20 mm in diameter against *S. sclerotiorum*. Also, *B. subtilis* and *B. cereus* are able to reduce the mycelial growth of *S. sclerotiorum* and to suppress the fungus in sunflower [32]. In the same way, *B. amyloliquefaciens* isolated from cucumber rhizosphere suppresses *S. sclerotiorum* mycelial growth by 72% [30]. *B. megaterium* strain MKB 135 significantly inhibits the *Septoria tritici* leaf blotch disease of wheat under field conditions [47]. *B. thuringiensis* and *B. subtilis* have been reported as effective biocontrol agents (BCAs) against *S. sclerotiorum* in several studies [1,3,15,17,20]. In this context, *E. cloacae* and *K. pneumoniae* strains possess multiple mechanisms for antagonistic action against *S. sclerotiorum* [24,48]. *Chryseobacterium* species are also commonly found in soil and water and their ability to suppress *S. sclerotiorum* [49] and other soilborne pathogens was reported [50,51].

These tomato-associated rhizobacteria were shown to act against *S. sclerotiorum* using non volatile and volatile compounds. Interestingly, pathogen growth inhibition due to volatile metabolites ranged between 24 and 54%. This indicates the important antifungal potential of the volatile organic compounds from the strain collection used in the current study. Also, volatile compounds of 7 cucumber-associated *B. amyloliquefaciens* isolates are shown able to prevent by more than 30% the mycelial growth of the fungus [30]. This result is in agreement with previous findings [52] where 76.59% decrease in *S. sclerotiorum* growth is obtained using volatile organic compounds. *B. subtilis* is able to produce volatile elements exhibiting antifungal activity toward *R. solani* and *B. cinerea* [53,54].

Bacterial antifungal volatiles may have the potential, as they can diffuse through the soil, to kill the overwintering sclerotia. Their main mode of action is their fungicidal effect on sclerotia through preventing them from germinating even under favorable conditions [13]. In fact, *S. sclerotiorum* spends 90% of its life cycle in soil as a sclerotia, able to survive up to 5 years in soil, and during their myceliogenic germination, the hyphae grow towards host roots and hypocotyls causing shoot wilt [55]. Interestingly, in the present work, all rhizobacterial strains had totally (100%) suppressed myceliogenic sclerotial germination, where bacterized sclerotia replated on fresh PDA plates failed to grow, but had decreased *S. sclerotiorum* mycelial growth by 37-57%. These findings are in agreement with previous reports [31] showing that isolate DF35 is able to inhibit sclerotial germination by 100%, but limits the mycelial growth by 50% only while the other bacteria, isolated from canola and soybean plants, cause 100% inhibition of mycelial growth and germination of sclerotia. Alterations in sclerotial germination and mycelial morphology are observed in the presence of lipopeptides-containing supernatants from *Bacillus* strains cultures [13]. Sclerotia treated by bacterial cells exhibit changes in color and less resistance with impaired consistency [21].

The control of the sclerotia is considered as a key in the control of *S. sclerotiorum* as sclerotial control would reduce the apothecial formation, through carpogenic germination, which would decimate ascospore production, the most important infection units in many crops and also would limit myceliogenic germination responsible of root infection. In this way, decreased or delayed germination of

sclerotia, achieved using this rhizobacteria collection, could be useful to reduce primary pathogen inoculum as noted with the lipopeptides producing *B. amyloliquefaciens* strains [13]. Similar effects have been reported by other authors on phytopathogenic fungi, suggesting that biosurfactant lipopeptides can traverse the fungal cell wall and induce serious alterations in plasma membrane permeability of active and resting structures [56,57].

In the present study, tomato cv. Rio Grande seed bacterization led to improved germination as compared to the untreated controls. Similar effects are reported on tomato seeds using *Burkholderia gladioli* pv. *agaricola* [58] and *Bacillus* spp. rhizobacterial strains [59].

The results of the *in vivo* screening of disease-suppressive effects revealed that the 25 rhizobacterial strains had inhibited pathogen development and reduced Sclerotinia Stem Rot severity on all inoculated and treated plants compared to the inoculated and untreated ones. The most effective strains in totally suppressing disease (i.e. having 0 as disease index) were *B. subtilis* B10 (KT921327), *E. cloacae* B16 (KT921429), *B. amyloliquefaciens* B13 (KT951658) and B15 (KT923051), and *B. thuringiensis* B2 (KU158884). Besides, all rhizobacteria-based treatments had significantly increased plant growth parameters i.e. plant height by 52-67%, aerial parts' fresh weight by 47-74% and root fresh weight by 66-88%. Thus, data from this study highlighted the additional growth-promoting effects exhibited by the rhizobacterial collection tested when challenged to tomato plants already infected with *S. sclerotiorum*. These results are in agreement with findings from various studies ensuring competitive yields while protecting plant health and soil [18,49,59]. Indeed, a successful biocontrol agent is generally equipped with several attributes which often promotes plant growth as efficiently as it inhibits fungal growth by efficient root colonization, phytohormone production and nutrient competition [60]. Other mechanisms of action are involved in PGP effects such as increased root permeability, improved capability to survive in strict competitive niche and root sites as well as through suppression ability of pathogenic microorganisms [61]. Members of *Bacillus* genus are among the beneficial bacteria mostly exploited as biopesticides [1,41], but to our knowledge, it is the first time that native *Bacillus* spp., *C. jejuense*, *E. cloacae*, and *K. pneumoniae* were used against *S. sclerotiorum* in Tunisia.

As shown in this study, among the tested rhizobacteria collection, the most promising strains combining disease-suppressive and growth-promoting abilities were *B. subtilis* B10 (KT921327) and B14 (KU161090), *B. thuringiensis* B2 (KU158884), *B. amyloliquefaciens* B13 (KT951658) and B15 (KT923051), and *E. cloacae* B16 (KT921429). Also, interestingly, *C. jejuense* B12 (KT921328) and *K. pneumoniae* B11 (KU158886) strains had also decreased Sclerotinia Stem Rot severity by 77%, and enhanced root growth by more than 80% and aerial part weight and plant height by more 50% compared to pathogen-inoculated control. These disease-suppressive and growth-promoting effects exhibited by these strains may be attributed to their ability to produce lipopeptide antibiotics, IAA and siderophores and to their capability to solubilize phosphate. Research into the mechanisms of plant growth promotion by bacteria has provided a greater understanding of the multiple facets of disease suppression by these biocontrol agents. In fact, PGPR produce a wide range of secondary compounds that may act as signals, allelochemicals, including metabolites, siderophores, antibiotics, volatile metabolites, enzymes, and others [60]. In fact, IAA production by saprotrophs and endophytes has been reported to be involved in growth promotion in various plants and, thus, could be responsible, at least in part, for the presently observed plant growth promotion [62,63]. Similar combined effects are displayed by *B. subtilis*

BN applied to chirpine seedlings where reduction in root rot symptoms caused by *M. phaseolina* in along with 43.6% and 93.45% increased root and shoot dry weight, respectively, are recorded as compared to the untreated control [64]. The use of bacteria as a soil treatment is more effective in suppressing disease than their use as a seed treatment [30]. In fact, the percentage of healthy plants in the presence of bacterial strains applied as soil treatment and *Sclerotinia* inoculum are significantly higher than those of pathogen-inoculated and untreated control plants. Significant growth promotion of *Arabidopsis* is also achieved using *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a strains [65]. Inoculation of plants with rhizobacteria could result in significant changes in seeds' germination and increases in various growth parameters such as plant height, root fresh weight and aerial part weight [66].

PGPR are commonly used as inoculants for improving growth and yield of agricultural crops and offers an attractive way to replace chemical fertilizers, pesticides, and supplements [67]. Suppression of Sclerotinia Stem Rot disease achieved, in the current study, using *Chryseobacterium* B12 strain is in agreement with previous findings [49] where *Chryseobacterium* spp. are reported to be putative biocontrol agents able to suppress *S. sclerotiorum* on tomato plants. *C. balustinum* tomato-associated rhizobacteria also shows PGP traits and improves aerial surface, aerial length and also the aerial parts and roots dry weights [18]. *Klebsiella* strains significantly increase shoot height and root length of inoculated wheat seedlings compared to the control [68]. Similarly, significant increases in shoot dry weight, plant height, and yield are recorded on tomato plants challenged with *B. amyloliquefaciens*, *B. subtilis* and *B. cereus* and other rhizobacteria (*Serratia marcescens*, *P. putida*, *P. fluorescens*) [69]. Also, the interaction of *Bacillus* spp. with potato seeds or vegetative parts show promising antagonism by virtue of producing siderophore and antibiotics against black scurf and stem canker diseases of potato caused by *R. solani*, thereby resulting in increased potato yield [70].

Conclusion

With increasing awareness about chemical fertilizers and pesticides, it is important to search for region-specific microbial strains with ability to act as a potential plant growth promoters and biocontrol agents. The current study provides strong evidence that tomato rhizospheric soils from tomato-growing regions of Tunisia yielded various isolates from *Bacillus*, *Enterobacter*, *Chryseobacterium*, and *Klebsiella* genera with plant growth-promoting traits and disease-suppression ability variable in a strain-specific manner. They could be useful as biofertilizers and biofungicides once their effectiveness proved under field conditions. Further investigations will be also focused on the effects of antagonist mixtures of the most effective strains against tomato rot diseases and plant growth.

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