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Evaluation of the Effectiveness of Tomato-Associated Rhizobacteria Applied Singly or as Three-Strain Consortium for Biosuppression of Sclerotinia Stem Rot in Tomato

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Abstract

In the present study, the capacity of three native tomato-associated rhizobacteria (*Bacillus subtilis* B2, *B. thuringiensis* B10, and *Enterobacter cloacae* B16) to suppress Sclerotinia Stem Rot in tomato and to improve growth was investigated in two tomato cultivars. The three bacterial strains were tested against *S. sclerotiorum* either singly or as consortium and their efficacy was compared to a fungicide control. All bacteria-based treatments were found to be more effective in suppressing disease than chemical fungicide on both cultivars and in both year trials. The disease-suppression and growth-promoting abilities of the treatments tested varied significantly depending on bacterial strains used, tomato cultivars grown, and year trial. Overall, all three strains suppressed the disease more effectively than the chemical fungicide. Indeed, for both year trials and cultivars combined, disease suppression potential, as compared to pathogen-inoculated and untreated control, ranged between 80.79 and 88.01% using the three-strain consortium relative to 70.00-82.07% achieved with single strains and 32.13-58.97% using fungicide. Plants grown in *S. sclerotiorum*-infected peat and challenged with the three-strain consortium were 38.36 to 80.95% taller than control ones whereas height increment noted using single strains and fungicide was of about 32.35-79.01 and 29.62-51.85%, respectively. Aerial parts and root fresh weights of pathogen-inoculated and treated plants were enhanced by 51.59-74.69% and 54.00-78.12% using mixed strains and by 39.12-76.83% and 42.02-77.01%, respectively, using single strains compared to 24.04-53.05 and 12.74-67.05% noted on chemically treated plants. The effect of the three biocontrol agents was also examined on the composition of microbial communities inhabiting the rhizosphere of tomato plants. Results of the single strand conformational polymorphism (SSCP)-based profiling revealed that rhizosphere communities differed between cultivars only. However, the introduction of *S. sclerotiorum* or biocontrol agents did not cause detectable perturbations in the composition of fungal and bacterial communities inhabiting roots of treated tomato plants.

Keywords: Biocontrol; Microbial community; Plant growth; *Sclerotinia sclerotiorum;* Strain-consortium; Tomato

Introduction

Tomato (*Solanum lycopersicum* L., formerly, *Lycopersicon esculentum* Mill.) is one of the most important vegetable crops worldwide [1]. In Tunisia, several fungal diseases are known to affect this crop during all stages of plant development resulting in severe damage in roots and/or crown, stems, leaves and fruits. Sclerotinia Root Rot, caused by *Sclerotinia sclerotiorum* (Lib.), is one of the most serious soilborne diseases of many vegetable crops including tomato [2]. This fungus is responsible for more than 60 diseases and survives in soil as sclerotia which germinate myceliogenically or carpogenically, depending on environmental conditions, leading to rotting of aerial parts of the plant in contact with soil [3].

Many strategies have been developed for Sclerotinia disease control such as cultural practices, chemical control, and soil solarisation but serious losses still occur largely because the effectiveness of these approaches is variable and often short lived. Furthermore, no genetic resistance toward this pathogen is currently available for tomato [4]. Such issues, as well as the necessity to reduce energy costs in farming and to develop more eco-compatible and more safe control methods, research efforts have focused on biological control using, among others, plant growth-promoting rhizobacteria (PGPR) [5]. PGPR can directly benefit plant growth through production of growth regulators, increasing nitrogen uptake, synthesis of phytohormones, solubilization of minerals, and iron chelation [6]. Some PGPR strains may also suppress soil borne pathogens by producing siderophores and antimicrobial metabolites or by competing for nutrients and/or niches [7]. Several biocontrol agents such as *Bacillus subtilis* [8], *B. thuringiensis* [9] and *Enterobacter cloacae* [10] have been used for *S. sclerotiorum* biocontrol.

Emerging strategies for plant disease management involve biological and integrated biological control by applying antagonistic microorganisms alone or in combination [11]. Single antagonistic strains often result in inconsistent disease control under field conditions and for overcoming such inconsistent performance, mixture of two or more bioagents, as biocontrol consortium, leads to more efficient

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disease control [12]. Mixed biocontrol agents (BCAs) have the potential to colonize more effectively the rhizosphere, to express more consistent beneficial traits under various soil conditions, and to control a wide range of plant pathogens than singly used agents due to their ability to produce various lipopeptide antibiotics [13].

In a previous study, we selected three fengycin- and/or bacillomycin-producing strains, i.e., *B. subtilis* B2, *B. thuringiensis* B10 and *Enterobacter cloacae* B16 based on their potential to suppress Sclerotinia Root Rot disease and to promote tomato growth [14]. This ability to produce antibiotics is reported to be a key tool by which PGPR strains can inhibit plant pathogens and suppress diseases. However, they exhibit broad spectrum activity [15] and thus, their impact is potentially not limited to the target fungal pathogens and may affect indigenous microbial community after release of BCAs [16]. Therefore, the knowledge of microbial ecology of the target habitat is necessary for accurate elucidation of the relationship occurring between released and indigenous or targeted pathogens. Therefore, the rhizosphere microbial diversity has been widely analyzed using common cultivation techniques but recently several DNA-based analyses, i.e. cultivation-independent methods such as Single Strand Conformational Polymorphism (SSCP), were developed and are being widely used [17].

In our previous studies, we have demonstrated that three selected strains out of 25 tested (namely *B. subtilis* B2, *B. thuringiensis* B10 and *Enterobacter cloacae* B16) exhibited strong biocontrol and biofertilizing effects when applied singly against Sclerotinia Stem Rot disease [14]. Moreover, these strains exhibited similar potentialities when applied either singly or as a three-strain consortium against Rhizoctonia Root Rot in two tomato cultivars tested over two year trials [18].

In the current investigation, we evaluated the capacity of these three rhizobacteria, applied singly or as consortium, to suppress Sclerotinia Stem Rot and to promote growth on two tomato cultivars compared to a chemical treatment. Furthermore, possible changes in rhizosphere microbial community upon biocontrol treatments tested was also investigated using Single Strand Conformational Polymorphism (SSCP) analysis.

Materials and Methods

Plant material and growth conditions

For biocontrol bioassays and elucidation of possible changes in rhizosphere microbial community, 21 day old tomato (cvs. Marmande and Rio Grande) seedlings were used. Seeds were surface-sterilized for 2 min into 2% sodium hypochlorite solution, washed thoroughly three times with sterile distilled water (SDW), and sown into disinfected dimpled plates containing sterile peat. Tomato seedlings were grown in a growth chamber at 13/11 h light/dark photoperiod and 21/18 ± 2°C light/dark temperature and regularly watered until being used.

Pathogen growth conditions and inoculum preparation

S. sclerotiorum isolate used in this study was originally isolated from tomato plants showing typical symptoms of Sclerotinia Stem Rot [14]. Cultures were grown on potato dextrose agar (PDA) medium supplemented with streptomycin sulfate at 300 mg/mL and stored at 4°C until use [19].

For inoculum production, ten PDA Petri plates (9 cm in diameter) showing full mycelium growth of pathogen, previously grown on PDA for 5-6 days at 28°C, were macerated using a blender in 1 L of SDW. Inoculum suspension was adjusted at 10^8 mycelial fragments /mL using a Malassez haemocytometer.

Rhizobacterial strains tested and inoculum preparation

Three rhizobacterial strains namely *Bacillus subtilis* B10 (KT921327), *B. thuringiensis* B2 (KU158884), and *Enterobacter cloacae* B16 (KT921429), selected out of 25 tested based on their ability to suppress Sclerotinia Stem Rot severity and to enhance tomato growth in a previous work [14]. These strains were identified and characterized in a previous study [20] and their main characters are provided in Table 1.

The strains stock cultures were maintained at -20°C in Luria Bertani (LB) broth amended with 15% glycerol. Before being used, stock cultures were cultured onto Nutrient Agar (NA) medium and incubated at 28°C for 24 h. A loop-full of each bacterial strain was injected into 300 mL of Nutrient Broth (NB) and grown in a rotary shaker at 175 rpm for 48 h at 28°C. After incubation, 300 mL of the obtained culture was diluted into 1 L of SDW and adjusted to approximately 10^8 cells /mL before being used for plant bacterization [21].

For preparation of mixed biocontrol formulation, equal volumes of each bacterial cell suspension were mixed and the three-strain consortium obtained was used for plant treatment.

Plant infection and bacterization

Tomato cvs. Rio Grande and Marmande seedlings, previously grown into dimpled plates containing sterile peat, were not watered two days prior to bioassay. Seedlings treatment was performed as substrate drench at the collar level using 30 mL of the bacterial cell suspension of either single strains or their consortium (10^8 cells/mL). One week post-bacterization, 30 mL of pathogen inoculum were poured at the same level to each seedling. One day after pathogen challenge, seedlings were transplanted into pots (16 cm in diameter) containing pathogen-infected peat [22].

Strains[a]	Lipopeptide production ability[b]		PGPR traits[c]		
	Fen A	Bac D	IAA[d]	P. Solubilization[e]	Siderophore production[f]
Bacillus thuringiensis B2 KU158884	-	+	+	+	++
B. subtilis B10 KT921327	+	+	+	+	+++
Enterobacter cloacae B16 KT921429	+	+	+	+	+++

[a] Molecular identification was performed by amplification and sequencing of 16S rRNA and *rpo*B genes [27]
[b] Lipopeptide production ability was confirmed by amplification and sequencing of genes encoding for Fengycin A (Fen A) and Bacillomycin D (Bac D) biosynthesis [27]
[c]PGPR traits: Plant growth promoting rhizobacteria traits
IAA: Indole-3-acetic acid production determined according to Ghodsalavi et al. [48] protocol
[e]P. solubilization: Phosphate solubilization ability assessed qualitatively using a modified Pikovskaya's agar medium containing tricalcium phosphate [49]
[f]Production of siderophore was assessed by spot inoculation onto Chrome Azurol S agar medium [50]. Production was scored as negative (−), low (+), middle (++) and high (+++)
All isolates were negative for hypersensitive reaction (HR) on tobacco leaves
Positive reaction (+); Negative reaction (–)

Table 1: Main characters of rhizobacterial strains used in this study and originally isolated from tomato rhizosphere.

Pathogen-inoculated seedlings watered with SDW only (water control) or treated with a commercial fungicide, i.e., Previcur Energy™ (632.6 g/L Propamocarb-Hcl+332.6 g/L Fosethyl-Al) applied at 0.5 mL/mL, were used as controls.

Pots were placed under greenhouse conditions (65% RH, 13/11 h light/dark photoperiod at 21 ± 2/18 ± 2°C light/dark temperature) till the end of the experiment. The whole experiment was repeated over two year trials (2012 and 2013).

Parameters noted

Two months pathogen challenge, plant height and aerial parts and roots fresh weights were recorded. Sclerotinia Stem Rot severity on roots was also evaluated 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 determined by dividing the number of diseased plants over the total number of plants used per individual treatment.

Impact of bacterial treatments on rhizosphere microbial community

DNA extraction from root samples: Root samples were taken from each individual treatment for analysis of eventual rhizosphere microbial community shifts occurring after seedling bacterization. Roots were cut into fragments (5 mm in length) and stored in a -20°C (1 g root segments per sample) until further use for microbial and molecular analyses.

Total DNA was extracted from 60 mg of root tissues according to Godon et al. [23] protocol with slight modifications. Samples were freeze-dried overnight at -80°C and lyophilized for 12 h. Six hundred µL of CTAB (1x) was added to each sample. After incubation at 65°C for 1 h, 400 µL of chloroform-isoamyl alcohol (24:1, v/v) was added to remove proteins, and shaken at 200 rpm for 10 min, and then samples were centrifuged at 13,000 rpm for 10 min/4°C. The aqueous phase was transferred into another tube, and 330 µL of cold isopropanol were added. Samples were then kept at -20°C overnight for DNA precipitation. After 10 min centrifugation at 13,000 rpm/4°C, the supernatant was discarded and 800 µL of ethanol 70% was added to wash the DNA. Once the ethanol discarded at 13,000 rpm/4°C for 10 min, the pellets were air-dried and suspended into 100 µL of SDW. DNA concentration was estimated using Nano-drop (ND-1000, Thermoscientific) and homogenized at a concentration of 10 ng/µL.

Analysis of rhizosphere fungal and bacterial community: For Capillary Electrophoresis-Single Strand Conformation Polymorphism (CE-SSCP) analysis of fungal and bacterial community, pairs of universal primers recognizing mitochondrial large-subunit rDNA (ML1/ML2) [24] gene and the variable regions V5-V6 of the 16S rRNA (799F/1115R) [25] were used, respectively. PCR amplification was performed on DNA samples from the 90 root samples collected. DNA was amplified by PCR in a PTC-100 thermocycler (MJ Research, Inc.) in a reaction mixture (30 µL final volume) consisting of 1 µL of DNA template (10 ng/µL), 2.5 µL of Pfu turbo buffer (10x), 2.5 µL de BSA at 10 µg/µL (BioLabs), 0.5 µL of $MgCl_2$ (50 mM), 1 µL of dNTP (10 mM), 0.5 µL of each primer, 0.5 µL of Pfu turbo (Stratagene), and 21 µL of SDW. The cycling conditions were as follows: enzyme activation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 30 s, hybridization for 30 s at 58°C for fungal and at 61°C for bacterial primers, extension at 72°C for 1 min, and final extension at 72°C for 10 min.

Genetic structure of fungal and bacterial community inhabiting the rhizosphere of rhizobacteria-treated tomato plants: The PCR products were visualized by 2% Tris-borate-EDTA agarose gel electrophoresis prior to SSCP analysis. The lengths of the fragments yielded by DNA's amplification were 250 bp for fungi (ML1/ML2) and 350 bp for bacteria. (799f/1115r). SSCP analyses were performed on an ABI Prism 3130 genetic analyzer (Applied Biosystems) using four 36 cm long capillary. One µL of a PCR product was mixed with 18.8 µL Hi-Di formamide (Applied Biosystems) and 0.2 µL of the internal standard DNA molecular weight marker Genescan 400 HD ROX (Applied Biosystems). The sample was then denatured for 5 min at 95°C and placed directly on ice for 10 min before being loaded onto the instrument.

Capillary Electrophoresis-Single Strand Conformation Polymorphism (CE-SSCP) is based on the electrophoretic mobility of single-stranded DNA fragments. This mobility is different according to their three-dimensional conformation. The samples were then allowed to co-migrate with the fluorescent size standard (GeneScan 400 ROX) to enable comparison of migration profiles between samples. Patterns were aligned with the Stat Fingerprints program [26] and studied by principal component analysis (PCA) using R software (version 2.15.2).

Structure and diversity analysis of microbial community: The characterization of the structure and the diversity of rhizosphere microbial community (fungi and bacteria) was performed based on profiles obtained using the CE-SSCP method according to Kimsé et al. [27] and Michelland et al. [26]. All readable molecular fingerprint profiles were aligned with the internal ROX ladder and normalized, to produce relative abundance data with the R package Stat fingerprints v1.3 software. This yielded a matrix in which root samples were indicated in rows, and fluorescence values (4866 scans) in columns. A fluorescence profile may be seen as a quantitative descriptor of the microbial assemblage of a sample. Bigger differences in fluorescence scans between profiles indicate a greater dissimilarity in composition between samples [26,27].

Diversity of rhizosphere microbial (fungi and bacteria) community was evaluated using Fingerprint molecular profiles studied using PCA in relation to environmental factors with R software (version 2.15.2, including FACTOMINER packages). In total, 72 samples were analyzed, i.e., 36 fungal amplicons (obtained using ML1 and ML2 primers) and 36 bacterial amplicons (obtained using primers 799f and 1115r).

Statistical Analysis

Data were analyzed using one-way analysis of variance and means separations were carried out according to Duncan's Multiple Range test at ($P \leq 0.05$). ANOVA analysis was performed using SPSS version 16.0 for all disease severity and plant growth parameters. The tests were conducted according to a completely randomized design where 6 treatments were tested and each individual treatment was replicated 12-15 times (i.e., 12-15 plants per individual treatment). The whole experiment was repeated twice (in 2012 and 2013 trials) and all data collected was presented in this paper.

The relationships between Sclerotina Stem Rot severity and plant growth parameters were compared using Pearson's correlation analysis at $P \leq 0.05$.

Analysis of the genetic structure of fungal and bacterial community inhabiting the rhizosphere war perfomed using PCA (R software version 2.15.2).

Results

The PGPR strains *B. thuringiensis* B2, *B. subtilis* B10, and *E. cloacae* B16 were tested singly or as three-strain consortium for their ability to suppress Sclerotinia Stem Rot and to improve plant growth on two tomato cultivars tested over two year trials (2012 and 2013).

Suppression of Sclerotinia Stem Rot using tomato-associated rhizobacteria

Sclerotinia Stem Rot incidence, noted 60 days post-planting and estimated based on the presence of root browning signs, varied from 46.66 to 100% depending on treatments tested, cultivars grown and year trials. Disease incidence noted in 2012 and 2013 (Table 2) ranged between 50-100% and 46.66-100%, respectively, for cv. Marmande compared to 60-100% and 73.33-100% recorded on cv. Rio Grande.

Based on their capacity to reduce disease severity, all rhizobacteria- and fungicide-based treatments had significantly decreased the root browning index as compared to *S. sclerotiorum*-inoculated and untreated control. All bacterial strains tested singly or as three-strain consortium, were found to be more effective in reducing disease severity than the fungicide on both cultivars and in both year trials (2012 and 2013). Data shown in Table 2 indicated that Sclerotinia Stem Rot severity, noted on cv. Marmande treated with bacterial strains was reduced by 82.01 to 88.01% in 2012 and by 72.24 to 86.98% in 2013 compared to 32.13 and 58.97%, respectively, achieved using fungicide. For cv. Rio Grande, disease index decrease noted using rhizobacteria-based treatments ranged between 70.0 and 81.75% in 2012 and between 70.26 and 80.79% in 2013 versus 50.00-45.53% obtained with chemical treatment.

Compared based on their respective ability to suppress Sclerotinia Stem Rot severity, efficacy of bacterial treatments depended upon bioagents used either singly or in combination, cultivars grown, and year trials. Results given in Table 2 showed that the three-strain consortium exhibited significantly similar effectiveness in decreasing disease severity as compared to single-strain-based treatments in both cultivars and year trials. Overall, combined data indicated slight difference in efficacy of consortium compared to single strains where disease suppression ranged between 80.79 and 88.01% with combined strains and between 70.00 and 82.07% using single-strain-based treatments.

Comparative plant growth enhancement using single or three-strain consortium

Single rhizobacteria strains and their three-strain consortium were assessed for their plant growth-promoting (PGP) abilities based on various growth parameters and their efficacy was compared to *S. sclerotiorum*-inoculated and untreated controls and to a fungicide-based treatment. ANOVA analysis revealed that all parameters depended significantly ($P \leq 0.05$) upon treatments tested, tomato cultivars used, and year trials. Their respective effects on each growth parameter were commented below.

Plant height

Data shown in Table 3 indicated that all three rhizobacteria, tested singly or as consortium, significantly ($P \leq 0.05$) augmented plant height of *S. sclerotiorum*-inoculated and treated plants over the untreated ones (Table 3). For cv. Marmande plants, height increment ranged from 75.07 to 80.95% in 2012 and from 32.35 to 44.75% in 2013 compared to 51.85 and 29.62%, respectively, achieved using fungicide treatment. However, for cv. Rio Grande, height increase varied from 70.97 to 76.51% in 2012 and from 47.4 to 49.94% in 2013 relative to 51.19 and 42.23%, respectively, obtained using commercial fungicide.

Compared to the three-strain consortium, strains applied singly exhibited significantly similar, lower or higher plant growth promoting (PGP) effect than their mixture depending on tomato cultivars grown and year trials. In 2012 bioassay, plant challenge using *B. thuringiensis* B2, *B. subtilis* B10 and the three-strain consortium led to significantly similar height increase, as compared to the inoculated and untreated control, on cvs. Marmande and Rio Grande (78.45-80.95% and 74.37-76.51%, respectively). However, in 2013 trial, the three-strain consortium exhibited similar PGP effect as singly used rhizobacterial strains on both cultivars tested.

Aerial parts fresh weight

Data given in Table 4 revealed that for both cultivars grown and both trials, all tomato plants inoculated with pathogen and treated with

Tomato cultivar Year trial	Marmande				Rio Grande			
	2012		2013		2012		2013	
Antagonistic treatment	**Disease incidence (%)[f]**	**Disease severity[g]**	**Disease Incidence (%)**	**Disease severity**	**Disease incidence (%)**	**Disease severity**	**Disease Incidence (%)**	**Disease severity**
S. sclerotiorum-inoculated control	100	4.17 a (0.0)[h]	100	4.07 a (0.0)	100	4.0 a (0.0)	100	3.80 a (0.0)
S. sclerotiorum+B.t.B2[a]	83.33	0.75 c (82.01)	86.66	1.13 bc (72.24)	80	1.07 c (73.25)	80	1.07 c (71.84)
S. sclerotiorum+B.s.B10[b]	83.33	0.75 c (82.01)	73.33	1.00 c (75.43)	80	1.20 c (70.0)	86.66	1.13 c (70.26)
S. sclerotiorum+E.c.B16[c]	58.33	0.75 c (82.01)	60	0.73 c (82.07)	73.33	1.07 c (73.25)	73.33	0.93 c (75.53)
S. sclerotiorum+B.t.B2+B.s.B10+E.c.B16[d]	50	0.5 c (88.01)	46.66	0.53 c (86.98)	60	0.73 c (81.75)	86.66	0.73 c (80.79)
S. sclerotiorum+Fungicide[e]	100	2.83 b (32.13)	93.33	1.67 b (58.97)	100	2.0 b (50.0)	100	2.07 b (45.53)

[a]B.t.B2: *Bacillus thuringiensis* B2 applied as single treatment
[b]B.s.B10: *B. subtilis* B10 applied as single treatment
[c]E.c.B16: *Enterobacter cloacae* B16 applied as single treatment
[d]B.t.B2+B.s.B10+E.c.B16: Three strains applied as consortium
[e]Fungicide-based treatment using Previcur Energy™ (632.6 g/L Propamocarb-Hcl+332.6 g/L Fosethyl-Al)
[f]Disease incidence was calculated for each individual treatment by dividing the number of symptomatic plants over the total number of plants
[g]Sclerotinia Root Rot severity was assessed using an arbitrary 0-5 scale where: 0=no symptom and 5=100% of root browning
[h]Values in parenthesis indicate the percentage (in %) of decrease in disease severity as compared to *S. sclerotiorum*-inoculated and untreated control
Bacterial treatments were applied as substrate drench at the collar level using 30 mL of bacterial cell suspension of either single strains or their consortium (10^8 cells/mL)
Values within each column followed by the same letter are not significantly different according to Duncan's Multiple Range test (at $P<0.05$)

Table 2: Sclerotinia Root Rot-suppressive ability of three tomato-associated rhizobacteria, applied singly or as consortium compared to fungicide and untreated controls, noted 60 days post-planting in two tomato cultivars tested over two year trials.

Tomato cultivar	Marmande		Rio Grande	
Year trial	2012	2013	2012	2013
Antagonistic treatment	Plant height (cm)	Plant height (cm)	Plant height (cm)	Plant height (cm)
S. sclerotiorum-inoculated control	8.08 b (0.0)[f]	62.87 c (0.0)	9.6 d (0.0)	49.80 c (0.0)
S. sclerotiorum+B.t.B2[a]	37.5 a (78.45)	92.93 b (32.35)	37.47 a (74.37)	95.27ab (47.73)
S. sclerotiorum+B.s.B10[b]	38.50 a (79.01)	99.8 b (37.0)	39.53 a (75.71)	94.67 ab (47.4)
S. sclerotiorum+E.c.B16[c]	32.42 a (75.07)	113.8 a (44.75)	33.07 b (70.97)	98.60 a (49.5)
S. sclerotiorum+B.t.B2+B.s.B10+E.c.B16[d]	42.42 a (80.95)	102.0 ab (38.36)	40.87 a (76.51)	99.47 a (49.94)
S. sclerotiorum+Fungicide[e]	16.78 b (51.85)	89.33 b (29.62)	19.67 c (51.19)	86.2 b (42.23)

[a]B.t.B2: *Bacillus thuringiensis* B2 applied as single treatment
[b]B.s.B10: *B. subtilis* B10 applied as single treatment
[c]E.c.B16: *Enterobacter cloacae* B16 applied as single treatment
[d]B.t.B2+B.s.B10+E.c.B16: Three strains applied as consortium
[e]Fungicide-based treatment using Previcur Energy™ (632.6 g/L Propamocarb-Hcl+332.6 g/L Fosethyl-Al)
[f]Values in parenthesis indicate the percentage (in %) of increase in plant height as compared to *Sclerotinia sclerotiorum*-inoculated and untreated control
Bacterial treatments were applied as substrate drench at the collar level using 30 mL of bacterial cell suspension of either single strains or their consortium (10^8 cells/mL)
Values within each column followed by the same letter are not significantly different according to Duncan's Multiple Range test (at $P<0.05$)

Table 3: Plant height increment obtained using three tomato-associated rhizobacteria, applied singly or as consortium compared to fungicide and untreated controls, noted 60 days post-planting in two tomato cultivars tested over two year trials.

Tomato cultivar	Marmande		Rio Grande	
Year trial				
	2012	2013	2012	2013
Antagonistic treatment	APFW[f] (g)	APFW (g)	APFW (g)	APFW (g)
S. sclerotiorum-inoculated control	5.08 c (0.0)[g]	23.70 f (0.0)	17.21 g (0.0)	14.71 d (0.0)
S. sclerotiorum+B.t.B2[a]	21.92 a (76.83)	44.90 cd (47.22)	36.19 c (52.44)	45.57 b (67.72)
S. sclerotiorum+B.s.B10[b]	20.45 a (75.16)	38.93 bc (39.12)	34.01 a (49.4)	43.96 a (66.54)
S. sclerotiorum+E.c.B16[c]	20.71 a (75.47)	45.01 ab (47.35)	30.28 b (43.16)	33.18 b (55.67)
S. sclerotiorum+B.t.B2+B.s.B10+E.c.B16[d]	20.07 a (74.69)	54.66 a (56.64)	35.55 a (51.59)	48.44 a (69.63)
S. sclerotiorum+Fungicide[e]	10.82 b (53.05)	31.20 cd (24.04)	23.85 c (27.84)	31.23 b (52.89)

[a]B.t.B2: *Bacillus thuringiensis* B2 applied as single treatment
[b]B.s.B10: *B. subtilis* B10 applied as single treatment
[c]E.c.B16: *Enterobacter cloacae* B16 applied as single treatment
[d]B.t.B2+B.s.B10+E.c.B16: Three strains applied as consortium
[e]Fungicide-based treatment using Previcur Energy™ (632.6 g/L Propamocarb-Hcl+332.6 g/L Fosethyl-Al)
[f]APFW: Aerial part fresh weight.
[g]Values in parenthesis indicate the percentage (in %) of increase in the aerial part fresh weight as compared to *Sclerotinia sclerotiorum*-inoculated and untreated control
Bacterial treatments were applied as substrate drench at the collar level using 30 mL of bacterial cell suspension of either single strains or their consortium (10^8 cells/mL)
Values within each column followed by the same letter are not significantly different according to Duncan's Multiple Range test (at $P<0.05$)

Table 4: Enhancement of aerial parts' growth obtained using three tomato-associated rhizobacteria, applied singly or as consortium compared to fungicide and untreated controls, noted 60 days post-planting in two tomato cultivars tested over two year trials.

bacterial strains either singly or in combination showed significant increase in their aerial part fresh weight (APFW) as compared to *S. sclerotiorum*-inoculated and untreated control ones. Table 4 indicated that for cv. Marmande, APFW increment ranged from 74.69 to 76.83% in 2012 and from 39.12 to 56.64% in 2013 compared to 53.05 and 24.04% (in 2012 and 2013, respectively) noted on plants treated chemically. However, for cv. Rio Grande plants, this parameter was enhanced by 43.16 to 52.44% in 2012 and by 55.67 to 69.63% in 2013, using single strain or the three-strain consortium, compared to 27.84 and 52.89% recorded on fungicide-treated plants.

Based on their comparative potential to enhance the aerial parts growth on cv. Marmande, *B. thuringiensis* B2, *B. subtilis* B10, and *E. cloacae* B16 showed significantly similar PGP effect as the three-strain consortium in 2012 trial whereas in 2013, *B. subtilis* B10 exhibited significantly lower PGP effect (39.12%) than the three-strain consortium and the two other strains (47.22-56.64%). However, on cv. Rio Grande, single-strain-based treatments using *B. thuringiensis* B2 and *B. subtilis* B10 had significantly similar effect on this parameter as consortium in both trials (2012 and 2013).

Roots fresh weight

Data provided in Table 5 indicated that using rhizobacterial strains either singly or as consortium, root fresh weight (RFW) was significantly ($P \leq 0.05$) augmented relative to *S. sclerotiorum*-inoculated and untreated control, and that their PGP effect varied upon tomato cultivars grown and year trials. For cv. Marmande, RFW was improved by 57.45 to 64.87% in 2012 and by 60.55 to 67.21% in 2013 versus 12.74 and 51.21%, respectively, noted on fungicide-treated plants. However, on cv. Rio Grande plants, RFW increment obtained using single strains or their consortium varied from 42.02 to 56.79% in 2012 trial and from 70.65 to 78.12% in 2013 relative to 40.07 and 67.05%, respectively, recorded on plants treated with fungicide.

Regarding their comparative capacity to increase the RFW of tomato plants already challenged with *S. sclerotiorum*, the three strains were shown to be as effective as their consortium on cv. Marmande in both trials (57.45-67.21%) whereas on cv. Rio Grande, *E. cloacae* B16 showed significantly lower PGB effect (42.02%) than the two other strains and combined bacterial treatment but in 2012 trial (52.78-56.79%) whereas in 2013 bioassay, this strain behaved significantly similar as *B. thuringiensis* B2 (70.65-75.0%) compared to 77.01 and 78.12% obtained using *B. subtilis* B10 singly and the combined treatment.

Correlation between Sclerotinia Stem Rot severity and plant growth parameters

For cv. Marmande data, Pearson's correlation analysis indicated that

Tomato cultivar Year trial Antagonistic treatment	Marmande		Rio Grande	
	2012	2013	2012	2013
	RFW[f] (g)	RFW (g)	RFW (g)	RFW (g)
S. sclerotiorum -inoculated control	1.37 b (0.0)[g]	1.01 c (0.0)	1.78 c (0.0)	0.86 d (0.0)
S. sclerotiorum+B.t.B2[a]	3.22 a (57.45)	3.08 a (67.21)	3.77 a (52.78)	3.44 ab (75.0)
S. sclerotiorum+B.s.B10[b]	3.90 a (64.87)	2.56 ab (60.55)	4.12 a (56.79)	3.74 a (77.01)
S. sclerotiorum+E.c.B16[c]	3.30 a (58.84)	3.02 a (66.56)	3.07 b (42.02)	2.93bc (70.65)
S. sclerotiorum+B.t.B2+B.s.B10+E.c.B16[d]	3.55 a (61.41)	3.08 a (67.21)	3.87 a (54.0)	3.93 a (78.12)
S. sclerotiorum+Fungicide[e]	1.57 b (12.74)	2.07 b (51.21)	2.97 b (40.07)	2.61 c (67.05)

[a]B.t.B2: *Bacillus thuringiensis* B2 applied as single treatment
[b]B.s.B10: *B. subtilis* B10 applied as single treatment
[c]E.c.B16: *Enterobacter cloacae* B16 applied as single treatment
[d]B.t.B2+B.s.B10+E.c.B16: Three strains applied as consortium
[e]Fungicide-based treatment using Previcur Energy™ (632.6 g/L Propamocarb-Hcl+332.6 g/L Fosethyl-Al)
[f]RFW: Root fresh weight
[g]Values in parenthesis indicate the percentage (in %) of increase in the root fresh weight as compared to *Sclerotinia sclerotiorum*-inoculated and untreated control
Bacterial treatments were applied as substrate drench at the collar level using 30 mL of bacterial cell suspension of either single strains or their consortium (10^8 cells/mL)
Values within each column followed by the same letter are not significantly different according to Duncan's Multiple Range test (at $P<0.05$)

Table 5: Enhancement of root growth obtained using three tomato-associated rhizobacteria, applied singly or as consortium compared to fungicide and untreated controls, noted 60 days post-planting in two tomato cultivars tested over two year trials.

plant height was significantly and negatively related to disease severity parameter in 2012 (r=-0.719; *P*=1.1242E-12) and 2013 (r=-0.594; *P*=6.7413E-10) trials. This indicates that the severest Sclerotinia Stem Rot symptoms adversely impacted plant growth leading to significant stunting relative to the pathogen-free control plants. Similar significant correlations were recorded between APFW and disease index in 2012 (r =-0.730; *P*=3.3653E-13) and 2013 (r=-0.384; *P*=1.8316E-4) trials. Also, RFW was also negatively linked to disease severity in 2012 (r=-0.778; *P*=8.5854E-16) and 2013 bioassays (r=-0.544; *P*=3.0757E-8).

For cv. Rio Grande, Pearson's correlation analysis also revealed similar significant correlations between disease severity and growth parameters as for cv. Marmande. Plant height was significantly and negatively related to disease index in 2012 (r=-0.700; *P*=1.5315E-14) and 2013 trials (r=-0.659; *P*=1.5722E-12). Also, significant and negative correlation was detected between APFW and disease severity both in 2012 (r=-0.673; *P*=3.5559E-13) and 2013 bioassays (r=-0.712; *P*=3.6876E-15). RFW was also negatively linked to Sclerotinia Stem Rot index in 2012 (r=-0.477; *P*=2.0197E-6) and 2013 (r=-0.632; *P*=2.3466E-11) trials.

This analysis indicated that the decrease in Sclerotinia Stem Rot severity on tomato plants, achieved using these rhizobacteria applied either singly or as consortium, was correlated to the observed aerial parts and root growth enhancement.

Genetic structure of microbial community colonizing the rhizosphere of treated tomato plants

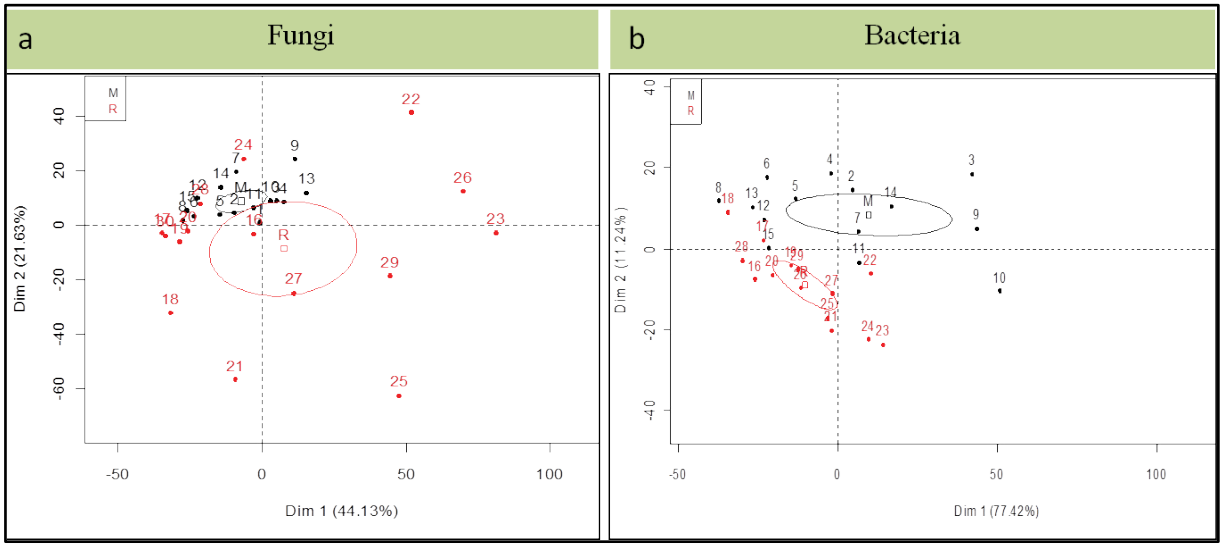
In total, 72 SSCP profiles (36 for bacteria and 36 for fungi) were generated from root samples collected from tomato cvs. Marmande and Rio Grande plants in 2013 trial. Based on the number of peaks and the relative height of the baseline, the SSCP profiles revealed complex microbial community (data not shown).

Principal Component Analyses (PCAs) were performed to compare the genetic structure of bacterial and fungal communities inhabiting the rhizosphere of both tomato cultivars infected with *S. sclerotiorum* and treated using rhizobacterial strains either singly or as consortium. The distributions of data from the different root samples on the principal plans generated by the PCA analysis for fungi and bacteria communities are provided in Figure 1. PCA eigenvalues indicate that the first two principal axes, Dim1 and Dim2, account for 65.7 and 88.6% of the total variability, respectively for fungi and bacteria. In both cases, microbial community varied only upon tomato cultivars grown. However, the introduction of *S. sclerotiorum* or rhizobacterial strains did not induce detectable shifts in the composition of fungal and bacterial communities inhabiting roots of treated tomato plants.

Discussion

The widespread use of synthetic chemicals as fungicides and fertilizers is a common practice in conventional farming in most parts of the world which threatens food safety and pollutes environment [28]. To attenuate or avoid their side effects, biological control is an alternative and proper choice for the management of various fungal soil borne diseases. Sclerotia-forming fungi and the serious diseases they cause are difficult to control due to their wide host range and the long survival of their resting structure. During the last decade, various microbial biological control agents (BCAs) have been identified for effective suppression of diseases incited by sclerotia-forming fungal pathogens [29]. Therefore, identifying novel BCAs with high antifungal potential is an attractive alternative for sustainable and safe agricultural practices. In a previous work [14], three strains (namely *B. thuringiensis* B2, *B. subtilis* B10 and *E. cloacae* B16), among 25 recovered from tomato rhizosphere, were selected for their potential to control Sclerotinia Stem Rot and to stimulate tomato growth. These strains also displayed potent efficacy when applied singly or as a three-strain consortium in suppressing Rhizoctonia Root Rot in two tomato cultivars tested over two year trials [18]. The present study was carried out in order to compare the relative efficacy of these selected strains when used singly or as three-strain consortium for bioprotection against Sclerotinia Stem Rot disease and plant growth promotion in the same tomato cultivars tested in both trials (2012 and 2013).

Mechanisms of action displayed by rhizobacteria during plant diseases control include mainly the release of secondary metabolites with antimicrobial activity [30], induced plant resistance [31] and growth promotion [32]. In the present study, seedling bacterization with *B. thuringiensis* B2, *B. subtilis* B10, and *E. cloacae* B16 and the three-strain consortium lowered disease severity on *S. sclerotiorum*-inoculated and treated plants. Their disease-suppression potential was proved on both tomato cultivars tested in two-year trials. These results are in agreement with our previous findings [14] where Rhizoctonia Root Rot suppression, as compared to the untreated controls, ranged



The variation (%) explained by each PCA axis is given in brackets. Ellipses represent the 95% confidence intervals calculated for each community

Figure 1: Principal Component Analysis (PCA) of fungal (a) and bacterial (b) communities inhabiting the rhizosphere of two tomato cultivars (R: Rio Grande (red) and M: Marmande (black)) based on SSCP profiles.

between 74.72 and 83.94% using three-strain mixture relative to 60.46-85.01% achieved using single strains. Moreover, these strains were previously shown to be Fengycin A- and/or Bacillomycin D-producing agents [20]. Thus, these strains applied singly or in combination, were shown able to suppress two tomato soil borne diseases. In fact, microorganisms acting through antibiosis were known to have a wide spectrum action [33]. Moreover, these three rhizobacterial strains were found to be more effective than the commercial fungicide, i.e., Previcur Energy™ (632.6 g/L Propamocarb-Hcl+332.6 g/L Fosethyl-Al), which is routinely used to protect horticultural crops and particularly for Sclerotinia Stem Rot control on pot-grown tomato. These findings are in agreement with several researches reporting on efficient control of the white mould pathogen using antagonistic bacteria [34] and particularly *Bacillus* spp. and *Enterobacter* spp. or and their by-products [8,10,35].

Based on disease severity and plant growth indicators, combination of rhizobacterial strains was found to be slightly more than or as effective as single strains in decreasing disease incidence and severity. The efficacy of these three strains differed depending on bioagents used, cultivar grown, and year trials. This effect was also noted when this same strain collection was used as single-strain-based treatments or as three-strain consortium for Rhizoctonia Root Rot biocontrol in the same tomato cultivars [14]. This may be explained by synergistic or antagonistic interaction between mixed BCAs that impacts their relative modes of action and the additive effects of their antifungal metabolites [36]. In fact, even if a single BCA has the ability to combat a plant pathogen [37], combination of antibiotic-producing strains may act synergistically in restricting growth and plant colonization abilities of targeted pathogens. Single use of BCAs for disease management might be also responsible for its inconsistent performance under field conditions resulting in inadequate site colonization and fluctuations in their abilities to release antimicrobial compounds [38]. This problem may be solved using mixtures of biocontrol strains thus mimicking natural environment [39]. Moreover, Jetiyanon and Kloepper [40] and Jetiyanon et al. [41] demonstrated that multi-strain consortia of PGPR strains have the potential to induce systemic resistance against various diseases than single strains.

The application of PGPR is a potentially attractive approach for disease management and improvement of crop yield. Results from the current study showed that all rhizobacteria-based treatments, used singly or as consortium, had significantly increased plant growth parameters (plant height, the aerial part and roots fresh weights) relative to the untreated controls (inoculated and pathogen-free). These findings are in accordance with previous results [20] where these strains had also enhanced growth of tomato plants of two tomato cultivars already challenged with *R. solani* while reducing Rhizoctonia Root Rot disease severity. Moreover, various other studies report on beneficial effects of PGPR strains used singly or in combination [9,42]. These additional fertilizing effects exhibited by the rhizobacterial collection tested when challenged to tomato plants already infected with *S. sclerotiorum* were in agreement with findings from various studies ensuring competitive yields while protecting plant and soil health [43]. Indeed, a potent BCA is generally equipped with several attributes which often promotes plant growth as it inhibits fungal growth through efficient root colonization, phytohormone production, and nutrient competition [44]. The strains tested were previously shown able to synthesize lipopeptide antibiotics, IAA and siderophores, and to solubilize phosphate [20]. The three-strain consortium tested in the current study showed synergistic effect in suppressing Sclerotinia Stem Rot and Rhizoctonia Root Rot diseases in tomato and in improving plant growth suggesting involvement of additive effects of their respective mechanisms of action.

Combinations of BCAs have the ability for more extensive colonization of rhizosphere volume, more consistent expression of their beneficial traits under various soil conditions, and for inhibiting a large number of plant pathogens than when applied singly [38]. Soil inoculation with high densities of viable and potent bioagents, for rapid rhizosphere colonization, would induce some shifts in the natural equilibrium of soil microbial communities [45]. Therefore, the assessment of the microbial community structure in the rhizosphere is considered critical to the successful and safe use of BCA strains. Moreover, several previous studies have shown that rhizosphere microbial community is influenced by plant species due to differences in root exudation and rhizodeposition in different root zones [46]. Thus,

in the current study, bacterial and fungal populations of roots removed from all tomato plants were investigated for two cultivars using CE-SSCP method (Capillary Electrophoresis single Strand Conformation Polymorphism). The comparison of products of cultivated isolates and microbial community as determined by SSCP analysis indicated that all treatments modalities were detected in the PCA community profiles. SSCP-community analysis performed for cvs. Marmande and Rio Grande had clearly demonstrated the presence of different patterns for both cultivars grown suggesting that each cultivar selected its own specific microbial community. Furthermore, SSCP analysis also revealed that no differences in genetic structure were observed when neither rhizobacteria treatment nor pathogen challenge was considered. These findings are in accordance with other studies based on rRNA gene profiling techniques and community-level physiological profiles which have also demonstrated that plant cultivars are more involved in the selection of their associated microbial communities in the rhizosphere than any other factor such as soil origin or release of bioagents [14,47]. Thus, these selected strains did not induce non-target effects on microbial community as demonstrated here based on SSCP analysis.

Conclusion

This study clearly demonstrated the beneficial effects of the three selected PGPR strains (namely *B. thuringiensis* B2, *B. subtilis* B10 and *E. cloacae* B16), applied singly or as three-strain consortium, in suppressing Sclerotinia Stem Rot and in improving plant growth on two tomato cultivars tested over two year trials. Moreover, results of the study revealed that all three strains suppressed the disease more effectively than the chemical fungicide. The best beneficial effects were observed in plants treated by the consortium, thus suggesting that the three strains interacted synergistically. In addition to the reduction in disease symptoms, plants treated by these rhizobacterial strains grew taller and had higher biomass. Examined for their effects on the composition of microbial communities inhabiting the rhizosphere of tomato plants, SSCP-community analysis performed for cvs. Marmande and Rio Grande had clearly revealed a variation in rhizosphere microbial community, assessed under controlled conditions, depending on grown tomato cultivars only. Thus, these strains, shown able to colonize roots, were expected to persist in the rhizosphere without inducing adverse shifts in indigenous populations but this hypothesis needs to be more confirmed under field conditions together with their relative effects on disease severity, growth and yield parameters.

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