

Biocontrol of Rhizoctonia Root Rot in Tomato and Enhancement of Plant Growth using *Rhizobacteria* Naturally associated to Tomato

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Abstract

In the present study, 25 rhizobacterial isolates, obtained from rhizosphere of healthy tomato plants collected from various tomato-growing sites in Tunisia, were tested *in vitro* and *in vivo* against *Rhizoctonia solani*. This bacterial collection, composed of isolates belonging to *Bacillus* spp., *Enterobacter cloacae*, *Chryseobacterium jejuense*, and *Klebsiella pneumoniae*, was assessed for its antifungal potential against *R. solani* the causative agent of Rhizoctonia Root Rot disease in various crops including tomato. Antifungal activity of diffusible and volatile metabolites derived from these isolates was tested against target pathogen using dual and distance culture bioassays, respectively. Growth inhibition rates, recorded after 5 days of incubation at 25°C, depended significantly upon tested bacterial isolates and screening methods and reached 34-59% and 18-45% for diffusible and volatile metabolites, respectively. The screening of disease-suppressive and plant growth-promoting abilities of these tomato-associated rhizobacteria showed 47-100% decrease in disease severity and significant increments in plant height by 62-76%, roots fresh weight by 53-86%, and aerial parts fresh weight by 34-67% compared to pathogen-inoculated and untreated control. *B. thuringiensis* B2 (KU158884), *B. subtilis* B10 (KT921327) and *E. cloacae* B16 (KT921429) were found to be the most efficient isolates in decreasing *R. solani* radial growth, suppressing disease severity, and enhancing plant growth.

Keywords: Antifungal metabolites; Biocontrol; Disease severity; Growth promotion; Mycelial growth; *Rhizoctonia solani*; Tomato

Introduction

Tomato (*Solanum lycopersicum* L.) is the second most important vegetable crop worldwide after potato based on grown areas [1]. In Tunisia, it is a strategic and an economically relevant crop. However, this crop is still threatened by serious wilting and root-rotting pathogens both in greenhouse and open-field growing systems [2,3]. The most widely grown tomato cultivars were susceptible to soilborne infections and especially to Rhizoctonia Root Rot disease caused by *Rhizoctonia solani* (Kühn). This pathogen is mostly known as a damping-off agent but is also responsible for collar and root rots and eventual death of severely diseased plants leading to significant crop yield loss [4-6].

Efficient disease control is difficult due to the various host range of the causative agent, the persistence of its resting structure (sclerotia) in soil, the lack of genetic resistance and to the limited efficacy of chemical fungicides [7]. Such issues have focalized research efforts on development of environmentally safe, long lasting and effective alternatives such as biological control [8].

Several biocontrol agents (BCAs) were reported to be effective in the bio-suppression of *R. solani* on various crops. The most efficient bacterial agents used for the biomanagement of Rhizoctonia Root Rot disease belonged mainly to the genera *Bacillus* [9-12], *Pseudomonas* [6], *Enterobacter* [13], *Serratia* [14], *Burkholderia* [12,15] and *Streptomyces* [16].

Among the group of BCAs, plant growth promoting rhizobacteria (or PGPR) have been widely used for the bio-suppression of various soilborne diseases [17]. In fact, PGPR strains can display disease-

suppressive effects against various crown, root and foliar diseases through direct inhibition of target pathogens or indirectly via the induction of systemic resistance (ISR) which is active throughout the entire plant [18-20]. PGPR-treated plants showed enhanced emergence potential and increased vegetative and root growth [17,21,22].

In our previous studies, a collection of 25 rhizobacterial isolates, obtained from rhizospheric soils collected around healthy tomato plants and belonging to *Bacillus*, *Chryseobacterium*, *Enterobacter*, and *Klebsiella* genera, was morphologically, biochemically, molecularly, and metabolically characterized [23] and screened for its capacity to suppress *Sclerotinia sclerotiorum* *in vitro* and *in vivo*. Interesting results were obtained where these isolates had significantly protected tomato plants from Sclerotinia Stem Rot disease and enhanced growth of pathogen-inoculated plants [24]. In the current investigation, the same collection of isolates will be assessed for its antifungal potential against *R. solani* mycelial growth and its capacity to suppress Rhizoctonia Root Rot disease and to enhance growth of infected plants.

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Materials and Methods

Tomato cultivar and growth conditions

Tomato plants cv. Rio Grande seedlings were used for all *in vivo* bioassays. Seeds were disinfected with 5% sodium hypochlorite during 2 min, rinsed thrice with sterile distilled water (SDW) and air-dried. They were sown in disinfected alveolus plates and maintained under greenhouse conditions ($30 \pm 4^\circ\text{C}$; 13/11 h light/dark photoperiod). Seedlings were regularly watered to avoid water stress.

Pathogen origin and growth conditions

R. solani isolate used in the present study was originally isolated from tomato plants exhibiting severe Rhizoctonia Root Rot infection. Pathogen cultures were gratefully provided by the Laboratory of Plant Pathology at the Regional Centre of Research on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia. Pathogen was grown onto Potato Dextrose Agar (PDA) medium amended with streptomycin sulfate (300 mg/L w/v) and incubated at 25°C for 5 days before use.

Rhizobacterial collection tested and growth conditions

The 25 bacterial isolates used in the current study were originally recovered from the rhizospheric soils of apparently healthy and vigorous tomato plants grown in various infested tomato fields. 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 such as IAA detection, siderophore production, phosphate solubilization. Their main traits were previously detailed [23].

Rhizobacterial stock cultures were stored at -20°C in Luria Bertani (LB) broth amended with 15% glycerol. Bacterial cultures used for the different tests were previously grown for 48 h onto Nutrient Agar (NA) and incubated at 28°C .

Suspensions of bacterial cells used for plant challenge were prepared as previously described and adjusted to approximately 10^8 cells/mL using a haemocytometer [24].

Screening of the antifungal potential of tomato-associated rhizobacteria against *Rhizoctonia solani*

The antifungal activity of the 25 rhizobacterial isolates against *R. solani* was screened *in vitro* using dual culture and distance culture bioassays for elucidating the suppressive effects of their diffusible and volatile compounds, respectively.

Dual culture assay

R. solani 5 day-old cultures were used for this bioassay. Agar plugs (5 mm in diameter) were cut using a sterile cork borer and placed at one side of a Petri plate (9 cm in diameter) containing PDA medium. At the opposite side, 10 μL of a bacterial cell suspension (10^8 cells/mL) were dropped into a well (5 mm in diameter) performed using sterile cork borer in Petri plates containing PDA. Control plates were challenged with pathogen plugs and bacterial suspension was replaced by a same volume of SDW. Plates were maintained at 25°C for 5 days. Three plates were used per each individual treatment. The diameter of pathogen colony and the inhibition zone were measured and the percentage of inhibition of pathogen growth was calculated as previously described [23].

Distance culture assay

Antifungal activity of volatile metabolites of the tomato-associated

rhizobacteria against *R. solani* was assessed using the distance culture assay also known as the sealed plate method. For this test, 10 μL of 48 h-old bacterial culture adjusted to 10^8 cells/mL were dropped into wells (5 mm in diameter) performed using sterile cork borer in Petri plates containing NA medium. A second PDA Petri plate was challenged with pathogen plug only (5 mm in diameter). Both half plates were wrapped together with parafilm to seal in the bacterial volatile compounds. For control plates, pathogen-challenged half plate was inverted over a half one containing NA only. The paired plates were incubated at 25°C for 5 days. Three plates were used per each individual treatment. After the incubation period, the diameter of pathogen colony was measured and the percentage of growth inhibition was calculated as previously described [24].

Assessment of Rhizoctonia Root Rot-suppressive and plant growth-promoting abilities

The ability of the 25 rhizobacterial isolates to limit the *in vivo* expression of *R. solani* and to enhance plant growth was screened based on pot experiments maintained under greenhouse conditions. Rhizobacteria and pathogen cultures were prepared as described above. Tomato cv. Rio Grande seedlings (at the two-true-leaf growth stage), grown in alveolus plates, were watered at the collar level with 30 mL of a suspension of bacterial cells (adjusted to 10^8 cells/mL). Seven days post bacterial treatment, 30 mL of *R. solani* inoculum (mycelial fragments) were poured at the same level to each seedling. Control seedlings were watered with SDW only. One day post pathogen challenge, seedlings were transplanted into pots (16 cm in diameter) filled with peat previously infected with 40 ml of fungal inoculum. A reminder bacterial treatment was performed 24 h post-transplanting [24]. Overall, the bioassay included a positive control (pathogen-free and rhizobacteria-free seedlings), a negative control (*R. solani*-inoculated and untreated seedlings) and 25 treatments consisting of tomato seedlings pathogen-challenged and individually treated with the tested 25 rhizobacterial isolates.

Two months after transplanting, tomato plants were uprooted and washed for eliminating the adhering peat. Three growth parameters (plant height and aerial parts and roots fresh weights) were recorded. Disease severity on collars and roots was estimated based on a 0-5 scale depending on root browning extent on the whole root system 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 [24]. Disease incidence was also calculated for each individual treatment by dividing the number of symptomatic plants over the total number of plants.

Statistical analysis

The results were subjected to one-way analysis of variance and means separations were carried out using the Duncan's Multiple Range test at $P \leq 0.05$. ANOVA was performed using SPSS version 16.0. Experiments were conducted according to a completely randomized design both for the *in vitro* (26 individual treatments, 3 replications) and the *in vivo* trials (27 individual treatments, 5 replications). Correlation analyses between Rhizoctonia Root Rot severity and plant growth parameters were carried out using Pearson's correlation analysis at $P \leq 0.05$.

Results

Antifungal activity of diffusible metabolites from tomato-associated rhizobacteria toward *R. solani*

ANOVA analysis indicated that the diameter of *R. solani* colony,

noted after 5 days of incubation at 25°C, depended significantly (at $P \leq 0.05$) upon bacterial treatments tested. In fact, data given in Table 1 showed that all the 25 rhizobacterial isolates had significantly (at $P \leq 0.05$) lowered pathogen mycelial growth over the control. The percentage of growth reduction, versus the untreated control, varied between 34.44 and 59.26% depending on isolates and exceeded 40% using 18 isolates out of the 25 tested.

B. thuringiensis B2 (KU158884), *B. subtilis* B10 (KT921327), *E. cloacae* B16 (KT921429) (Figure 1) and *B. subtilis* B6 (KT921427) isolates were found to be the most active in inhibiting *R. solani* radial growth by 48.89-59.26%.

This assay also showed that some tested rhizobacterial isolates led to the formation of inhibition zones when dual cultured with *R. solani*.

Dimension of this zone ranged between 3 and 10.3 mm depending on isolates and was more than 5 mm when *R. solani* was dual-cultured with 13 out of the 25 isolates tested (Table 1). The largest inhibition zones, of about 8.3-10.3 mm, were induced by *E. cloacae* B16 (KT921429), *B. subtilis* (B14), and *B. megaterium* B24 (KT923048).

Antifungal activity of volatile metabolites from tomato-associated rhizobacteria toward *R. solani*

Data analysis revealed that the diameter of pathogen colony, recorded after 5 days of incubation at 25°C, varied significantly ($P \leq 0.05$) upon bacterial treatments tested. In fact, as shown in Table 1, *R. solani* growth was lowered by 18.52 to 45.37% over control due to the inhibitory effects of volatile metabolites released by the rhizobacterial

Bacterial treatment	Isolate	Diffusible metabolites			Volatile metabolites	
		Colony diameter (mm)	Growth Inhibition (%)	Inhibition zone (mm)	Colony diameter (mm)	Growth Inhibition (%)
<i>Bacillus megaterium</i>	B1	51.0 ^{bc}	43.34	3.3 ^{cd}	54.67 ^g	39.26
<i>B. thuringiensis</i>	B2	36.67 ^d	59.26	5.3 ^{abc}	50.0 ^g	44.44
<i>Enterobacter cloacae</i>	B3	49.5 ^{bc}	45	4.7 ^{bcd}	57.5 ^{defg}	36.11
<i>E. cloacae</i>	B4	53.67 ^{bc}	40.37	4.0 ^{bcd}	62.83 ^{bcddef}	30.18
<i>B. megaterium</i>	B5	52.83 ^{bc}	41.3	4.7 ^{cd}	66.83 ^{bcdde}	25.74
<i>B. subtilis</i>	B6	46.0 ^{cd}	48.89	3.3 ^{bcd}	56.67 ^{defg}	37.03
<i>B. amyloliquefaciens</i>	B7	50.0 ^{bc}	44.44	4.0 ^{bcd}	67.5 ^{bcd}	25
<i>B. subtilis</i>	B8	48.33 ^{bc}	46.3	4.3 ^{bcd}	64.17 ^{bcddef}	28.7
<i>B. amyloliquefaciens</i>	B9	59.0 ^b	34.44	6.0 ^{abc}	60.33 ^{cdef}	32.96
<i>B. subtilis</i>	B10	36.67 ^d	59.26	5.7 ^{abc}	49.17 ^g	45.37
<i>Chryseobacterium jejuense</i>	B11	52.0 ^{bc}	42.22	5.3 ^{abc}	64.5 ^{bcddef}	28.34
<i>Klebsiella pneumoniae</i>	B12	50.5 ^{bc}	43.89	3.3 ^{cd}	63.5 ^{bcddef}	29.44
<i>B. amyloliquefaciens</i>	B13	48.83 ^{bc}	45.74	5.0 ^{bcd}	63.33 ^{bcddef}	29.63
<i>B. subtilis</i>	B14	56.17 ^{bc}	37.6	9.0 ^{bc}	60.83 ^{cdef}	32.4
<i>B. amyloliquefaciens</i>	B15	56.83 ^{bc}	36.85	6.0 ^{abc}	62.0 ^{cdef}	31.11
<i>E. cloacae</i>	B16	37.0 ^d	58.89	10.3 ^a	49.17 ^g	45.37
<i>B. subtilis</i>	B17	58.83 ^b	34.63	4.0 ^{bcd}	60.5 ^{cdef}	32.78
<i>B. amyloliquefaciens</i>	B18	56.0 ^{bc}	37.78	7.0 ^{abc}	69.17 ^{bc}	23.15
<i>B. subtilis</i>	B19	53.83 ^{bc}	40.19	6.7 ^{abc}	55.83 ^{efg}	37.96
<i>B. subtilis</i>	B20	50.83 ^{bc}	43.52	4.0 ^{bcd}	62.67 ^{bcddef}	30.37
<i>B. amyloliquefaciens</i>	B21	50.33 ^{bc}	44.07	7.3 ^{abc}	73.33 ^b	18.52
<i>B. amyloliquefaciens</i>	B22	51.0 ^{bc}	43.34	6.0 ^{abc}	63.83 ^{bcddef}	29.07
<i>B. thuringiensis</i>	B23	58.33 ^b	35.19	4.7 ^{bcd}	65.0 ^{bcddef}	27.78
<i>B. megaterium</i>	B24	54.17 ^{bc}	39.81	8.3 ^{abc}	65.0 ^{bcddef}	27.78
<i>B. subtilis</i>	B25	53.67 ^{bc}	40.37	3.0 ^{cd}	63.83 ^{bcddef}	29.07
Untreated control		90.0 ^a	0	0.0 ^d	90.0 ^a	0

Table 1: Effects of diffusible and volatile metabolites released by tomato-associated rhizobacteria against *Rhizoctonia solani* growth noted after 5 days of incubation at 28°C. For each parameter, values followed by the same letter are not significantly different according to Duncan's Multiple Range test (at $P \leq 0.05$).



str. B2: *Bacillus thuringiensis* (KU158884); str. B10: *B. subtilis* (KT921327); str. B16: *Enterobacter cloacae* (KT921429).

Figure 1: Inhibition of *Rhizoctonia solani* mycelial growth induced by three tomato-associated rhizobacterial isolates noted after 5 days of incubation at 25°C as compared to the untreated control.

isolates tested. Pathogen growth decrease exceeded 30% with 13 out of the 25 tested. Volatiles from *E. cloacae* B16 (KT921429), *B. subtilis* B10 (KT921327), *B. thuringiensis* B2 (KU158884) and, at a lesser extent, those from *B. megaterium* B1 (KU168423), *B. subtilis* B19 (KT921430), and *E. cloacae* B3 (KT923049) were the most effective against *R. solani* leading to 36-45% lower radial growth relative to control.

Suppression of Rhizoctonia Root Rot disease using tomato-associated rhizobacteria

Disease incidence, calculated 60 days post-transplanting and estimated based on the presence of typical root browning symptoms whatever their levels of extent, varied from 0 to 100% depending on bacterial isolates used for seedling treatments (Table 2). Higher disease incidence (100%) records seemed to be more associated to root browning indexes ranging between 1 and 2.4.

Rhizoctonia Root Rot severity depended significantly ($P \leq 0.05$) upon tested bacterial treatments. As shown in Table 2, this parameter ranged from 0 to 2.4 (using 0-5 scale) for all rhizobacteria-based treatments and these disease severity scores were significantly ($P \leq 0.05$) lower than that recorded on pathogen-challenged and untreated control plants (disease index 4.6).

It should be highlighted that disease index values did not exceed 1 on tomato plants treated with 19 rhizobacterial isolates and were less than 0.5 with 13 out of the 25 tested indicating approximately total

suppression of disease development. Moreover, compared to *R. solani*-inoculated and untreated control, 21 out of the 25 tested isolates led to more than 70% decrease in disease severity and this percent ranged between 47 and 53% for the four remaining ones (Table 2). These results demonstrated the capacity of the rhizobacterial collection tested to decrease Rhizoctonia Root Rot development and severity.

It should be mentioned that, interestingly, isolates *B. thuringiensis* B2 (KU158884), *B. subtilis* B8 (KU158885), *B. subtilis* B10 (KT921327), *E. cloacae* B16 (KT921429), and *B. amyloliquefaciens* B21 (KT923047) had totally suppressed Rhizoctonia Root Rot disease. Furthermore, treatments of *R. solani*-inoculated plants using *B. megaterium* B1 (KU168423) and B5 (KT923054), *E. cloacae* B3 (KT923049) and B4 (KT923050), *B. subtilis* B6 (KT921427), B14 (KU161090), B17 (KT923055), and B19 (KT921430), *B. thuringiensis* B23 (KT923056), *B. amyloliquefaciens* B7 (KT921428) and B15 (KT923051) had significantly limited Rhizoctonia Root Rot severity by 86-96% relative to pathogen-inoculated and untreated control. *K. pneumoniae* B12 (KT921328) and *C. jejuense* B11 (KU158886) lowered disease severity by 73 and 83%, respectively, if compared to *R. solani*-inoculated and untreated control (Table 2).

These results indicate that this collection of native tomato-associated rhizobacteria exhibited variable and strong disease suppressive abilities.

Bacterial treatment	Isolate	Disease Incidence (%)	Disease severity	Root fresh weight (g)	Aerial part fresh weight (g)	Plant height (cm)
<i>Bacillus megaterium</i>	B1	40	0.4 ^{gh} (91.30) ¹	9 ^{ab} (84.44) ²	20.4 ^{cd} (53.93) ²	56 ^{bcd} (72.5) ²
<i>B. thuringiensis</i>	B2	0	0.0 ^h (100.0)	10.2 ^a (86.27)	25.4 ^{ab} (62.99)	63 ^{ab} (75.56)
<i>Enterobacter cloacae</i>	B3	60	0.6 ^{efgh} (86.95)	5.3 ^{defghi} (73.58)	15.8 ^{efgh} (40.51)	46 ^{ghij} (66.53)
<i>E. cloacae</i>	B4	60	0.2 ^{gh} (95.65)	5.5 ^{defghi} (72.55)	18.1 ^{defgh} (48.06)	50 ^{defghi} (69.2)
<i>B. megaterium</i>	B5	40	0.4 ^{gh} (91.30)	4.8 ^{ghijk} (70.83)	17.1 ^{defgh} (45.02)	52 ^{defgh} (70.39)
<i>B. subtilis</i>	B6	20	0.6 ^{efgh} (86.95)	5.1 ^{efghi} (74.55)	15.7 ^{gh} (40.12)	53 ^{defgh} (70.94)
<i>B. amyloliquefaciens</i>	B7	60	0.6 ^{efgh} (86.95)	5.4 ^{defghi} (74.07)	16.8 ^{defgh} (44.04)	49 ^{efghij} (68.57)
<i>B. subtilis</i>	B8	0	0.0 ^h (100.0)	6.7 ^{cde} (79.10)	22.3 ^{bc} (57.85)	58 ^{abcd} (73.45)
<i>B. amyloliquefaciens</i>	B9	100	1 ^{ef} (78.26)	5 ^{ghij} (72.0)	18.5 ^{cdefg} (49.18)	52 ^{defgh} (70.38)
<i>B. subtilis</i>	B10	0	0.0 ^h (100.0)	9.6 ^a (85.42)	26 ^{ab} (63.84)	61 ^{abc} (74.75)
<i>Chryseobacterium jejuense</i>	B11	60	0.8 ^{efg} (82.61)	5.5 ^{defghi} (74.55)	18.5 ^{cdefg} (49.19)	52 ^{defgh} (70.38)
<i>Klebsiella pneumoniae</i>	B12	100	1.2 ^{de} (73.91)	6.5 ^{cdef} (78.46)	18.2 ^{cdefgh} (48.35)	56 ^{bcd} (72.5)
<i>B. amyloliquefaciens</i>	B13	100	2.4 ^{bc} (47.82)	3.4 ^{klm} (58.82)	14.4 ^{ghi} (34.72)	43 ^{hij} (64.18)
<i>B. subtilis</i>	B14	40	0.4 ^{gh} (91.30)	4.4 ^{hijk} (68.18)	17.8 ^{cdefgh} (47.19)	48 ^{efghij} (67.92)
<i>B. amyloliquefaciens</i>	B15	20	0.2 ^{gh} (95.65)	7.8 ^{bc} (82.05)	21 ^{cd} (55.23)	55 ^{bcd} (72.0)
<i>E. cloacae</i>	B16	0	0.0 ^h (100.0)	10 ^a (86.0)	28.7 ^a (67.25)	65 ^a (76.31)
<i>B. subtilis</i>	B17	20	0.2 ^{gh} (95.65)	5.1 ^{efghi} (72.55)	18.9 ^{cdef} (50.26)	51 ^{defgh} (69.80)
<i>B. amyloliquefaciens</i>	B18	100	2.2 ^{bc} (52.17)	3.6 ^{klm} (61.11)	9.3 ^k (-1.07)	42 ^j (63.33)
<i>B. subtilis</i>	B19	60	0.4 ^{gh} (91.30)	6.4 ^{cdefg} (78.12)	18.9 ^{cdef} (50.26)	55 ^{bcd} (72.0)
<i>B. subtilis</i>	B20	100	1.8 ^{cd} (60.87)	4.2 ^{hijkl} (66.67)	10 ^{jk} (6.0)	46 ^{ghij} (66.52)
<i>B. amyloliquefaciens</i>	B21	0	0.0 ^h (100.0)	6.8 ^{cd} (79.41)	20.3 ^{cde} (53.67)	58 ^{abcd} (73.45)
<i>B. amyloliquefaciens</i>	B22	100	2.4 ^{bc} (47.83)	3 ^m (53.33)	11 ^{kl} (14.55)	42 ⁱ (63.33)
<i>B. thuringiensis</i>	B23	60	0.4 ^{gh} (91.30)	5.8 ^{defgh} (75.86)	19.7 ^{cdef} (52.29)	57 ^{bcd} (72.98)
<i>B. megaterium</i>	B24	80	0.8 ^{efg} (82.61)	4.1 ^{ijkl} (65.85)	17.9 ^{cdefgh} (47.48)	54 ^{cdefg} (71.48)
<i>B. subtilis</i>	B25	100	2.4 ^{bc} (47.83)	2.3 ^{mn} (39.13)	19.9 ^{cdef} (52.76)	41 ⁱ (62.44)
Untreated control	-	40	0.4 ^{gh} (91.30)	2.9 ^{lm} (51.73)	13.8 ^{hij} (31.88)	32 ^k (51.87)
<i>R. solani</i> - inoculated control	-	100	4.6 ^a	1.4 ⁿ (0)	9.4 ^k (0.0)	15.4 ^l (0)

Table 2: Effects of tomato-associated rhizobacteria on incidence and severity of Rhizoctonia Root Rot disease and growth of tomato plants noted 60 days post-planting. Rhizoctonia Root Rot severity was assessed using a 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= 100% of root browning [24].

1: Values in parenthesis indicate the percentage (in %) of decrease in disease severity as compared to *R. solani*- inoculated and untreated control plants.

2: Values in parenthesis indicate the percentage (in %) of increase in plant growth parameters as compared to *R. solani*- 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 $P \leq 0.05$).

Improvement of growth of *R. solani*-inoculated tomato plants using tomato-associated rhizobacteria

The 25 bacterial isolates, naturally associated to tomato, were screened for their plant growth-promoting effects based on various growth parameters and their data were compared to those of the untreated control plants (*R. solani*-inoculated or disease-free controls). ANOVA analysis indicated that the plant height and the aerial parts and roots fresh weights depended significantly ($P \leq 0.05$) upon tested bacterial treatments. Their comparative abilities to promote growth of above- and below-ground plant parts were detailed below.

Plant height increase

All the rhizobacterial isolates had significantly ($P \leq 0.05$) augmented the plant height of *R. solani*-inoculated and treated plants over the inoculated and untreated ones (Table 2). This increase ranged between 62.44 and 76.31% and exceeded 70% using 15 out of the 25 isolates tested.

The highest plant height increments, of about 73-76% compared to the inoculated and untreated control, were recorded on pathogen-inoculated plants treated with *E. cloacae* B16 (KT921429), *B. thuringiensis* B2 (KU158884), *B. subtilis* B10 (KT921327) and B8 (KU158885), and *B. amyloliquefaciens* B21 (KT923047). Moreover, treatments of tomato plants using these four isolates led to significant enhancement of their plant height by 51, 49, 47, and 45%, respectively, compared to disease-free and untreated control (Table 2).

Aerial parts fresh weight increase

Data given in Table 2 showed that plant treatment with the majority of tested isolates, excluding two isolates of *B. amyloliquefaciens* namely B18 (KT923052) and B22 (KT923053) and one isolate of *B. subtilis* B20 (KT921431), led to significant ($P \leq 0.05$) increase in the aerial parts fresh weight relative to *R. solani*-inoculated and untreated control. Enhancements recorded in the aerial parts fresh weight ranged between 34.72 and 67.25% depending on tested bacterial treatments and exceeded 50 and 60% using 11 and 3 isolates out of the 25 tested, respectively.

Based on their capacity to increase the aerial part growth of tomato plants already infected with *R. solani*, *E. cloacae* B16 (KT921429), *B. subtilis* B10 (KT921327), and *B. thuringiensis* B2 (KU158884) were

found to be the most promising PGPR candidates generating 67.25, 63.84, and 62.99% increase in this parameter over the inoculated control, respectively. Moreover, increments in the fresh weight of the aerial parts allowed by these isolates were significantly higher than that recorded on pathogen-free and untreated control plants.

Plants treated with *E. cloacae* B16 (KT921429), *B. subtilis* B10 (KT921327), and *B. thuringiensis* B2 (KU158884) showed significant increments in the aerial parts growth by 52, 47, and 46%, respectively, compared to disease-free control and untreated control (Table 2).

Roots fresh weight increase

Data presented in Table 2 revealed that the majority of the tested isolates, excepting *B. subtilis* B25 (KU161091), had significantly ($P \leq 0.05$) improved root development. In fact, root fresh weight increment, compared to pathogen-inoculated and untreated control plants, ranged from 53.33 to 86.27% depending on tested bacterial treatments and reached up to 70% using 18 isolates.

The greatest root growth-promoting effects, expressed by more than 84% increase in the root fresh weight, were obtained using *B. thuringiensis* B2 (KU158884), *E. cloacae* B16 (KT921429), *B. megaterium* B1 (KU168423), and *B. subtilis* B10 (KT921327) (Figure 2). Furthermore, plants infected with *R. solani* and treated with these four isolates exhibited 68-71% significantly higher root growth relative to pathogen-free and untreated control ones. This indicates that these tomato-associated bacterial isolates have additionally bio-fertilizing benefits.

Correlation between Rhizoctonia Root Rot severity and plant growth parameters

Pearson's correlation analysis demonstrated that plant height was significantly and negatively related to root browning index ($r = -0.773$; $P = 4.9843 \text{ E-}28$) indicating that increased disease severity led to plant stunting if compared to pathogen-free control ones. Similar correlations were noted between the fresh weights of the aerial parts ($r = -0.608$; $P = 5.5636 \text{ E-}15$) and roots ($r = -0.675$; $P = 2.8624 \text{ E-}19$) and disease severity.

This analysis indicated that the lowered Rhizoctonia Root Rot severity on tomato plants, allowed using rhizobacteria-based treatments, was linked to the registered growth promotion.



R. solani + str.B16 *R. solani* + str.B10 *R. solani* + str. B2 Untreated-control *R. solani* -inoculated control

str. B2: *Bacillus thuringiensis* (KU158884); str. B10: *B. subtilis* (KT921327); str. B16: *Enterobacter cloacae* (KT921429).

Figure 2: Suppression of Rhizoctonia Root Rot severity and increased root growth in tomato cv. Rio Grande achieved using three tomato-associated rhizobacterial isolates compared to *R. solani*-inoculated control and to disease-free untreated control noted 60 days post-transplanting.

Discussion

Recently, rhizobacteria have gained more attention because of their successful ability to colonize roots and to the broad spectrum of their metabolites involved in disease biocontrol and/or growth enhancement such as antibiotics, lytic enzymes, siderophore, and phytohormones [17,25-27]. In the present study, 25 rhizobacterial isolates, naturally associated to tomato plants and recovered from rhizospheric soils removed from tomato-producing sites Tunisia, were assessed against *R. solani*. This same rhizobacteria collection was previously tested and was shown able to inhibit *S. sclerotiorum* mycelial growth and myceliogenic germination of its sclerotia, to suppress Sclerotinia Stem Rot disease and to improve tomato growth [24].

Based on *in vitro* findings, diffusible and volatile metabolites from tested rhizobacterial isolates displayed antifungal activity against *R. solani* where *B. thuringiensis* B2, *B. subtilis* B6, *B. subtilis* B10, and *E. cloacae* B16 (KT921429) were found to be the most bioactive agents based on both dual and distance culture assays. According to Adesina et al. [28], the root-associated bacteria have an antagonistic potential towards *Rhizoctonia* spp. In fact, used as whole cell suspensions or cell-free culture filtrates, they displayed suppressive effects against root rot disease of tomato caused by *R. solani*. *Bacillus* spp. have been reported as effective biocontrol agents against *R. solani* in several other studies [10,29-31]. These findings are also in agreement with previous studies reporting the capacity of *B. subtilis* and *B. amyloliquefaciens* to control various fungal plant pathogens including *R. solani* using diffusible and/or volatile metabolites [32-36].

Antibiotic production by bacterial antagonists is an essential component in the biological control of fungal phytopathogens [37] and cyclic lipopeptide antibiotics, in particular, are able to suppress various phytopathogenic fungi including *R. solani* [38,39]. This antibiotic production ability within *Bacillus* spp. and their extensive uses as biocontrol agents have been reported in many reviews [40,41]. In fact, *B. subtilis* and *B. thuringiensis* synthesized at least five lipopeptide antibiotics including bacillomycin [10,42]. However, Mandal et al. [43] demonstrated that *E. cloacae* can produce kurstakin, iturin, surfactin and fengycins probably involved in its antifungal activity displayed toward *R. solani*. In fact, *E. cloacae* isolates tested in the current investigation were previously demonstrated able to produce fengycins A and/or bacillomycin D [23]. *Chryseobacterium* species were frequently encountered in soils and were effective against various soilborne pathogens [44,45]. However, their growth inhibitory effects vary depending on species and isolates. *C. jejuense* B11 used in the present work was previously found to be a bacillomycin D-producing agent [23]. However, in other studies [45,46], *C. wanjuae* KJ9C8 and *Chryseobacterium* species were found able to produce other antifungal compounds like hydrogen cyanide (or HCN) but not antibiotics.

The *in vivo* screening of the ability of the 25 rhizobacterial isolates to suppress Rhizoctonia Root Rot severity revealed that this bacterial collection contained interesting biocontrol agents. The most effective isolates allowing total disease suppression (i.e. having 0 as disease index) were *B. thuringiensis* B2, *B. subtilis* B10, *E. cloacae* B16, *B. subtilis* B8, and *B. amyloliquefaciens* B21. It should be highlighted that the isolates B2, B10 and B16 were previously found to be the most effective in suppressing Sclerotinia Stem Rot in tomato and in promoting plant growth [24]. Moreover, in the present investigation, *B. subtilis* B8 and *B. amyloliquefaciens* B21 isolates had totally suppressed Rhizoctonia Root Rot and, interestingly, had also enhanced by more than 70% the root fresh weight and plant height. However, in their *in vitro* screening, they had significantly decreased *R. solani* radial growth

by 44-46 and 19-29%, relative to the untreated control, using dual and distance culture bioassays compared to 58-59 and 45% achieved using the three most effective isolates. These both isolates were previously shown to be fengycins A- and bacillomycin D-producing agents and also able to produce IAA and to solubilize phosphate [23]. Thus, these properties may explain their *in vivo* efficacy but further investigations are still required to elucidate their probable unknown features. Overall, all tested isolates were efficient in controlling Rhizoctonia Root Rot but with a varying degree. In fact, in our previous study, among the rhizobacterial isolates screened for detection of fengycins A and bacillomycin D biosynthesis genes, 20 were able to produce at least one of these antibiotics and 15 isolates were positive for both antibiotic biosynthesis genes [23]. This finding may explain the recorded disease suppression achieved using these isolates.

During the ten last years, several workers underlined the ability of bacterial isolates belonging mainly to *Pseudomonas* [6,47,48] and *Bacillus* genera [9-12,49,50] to control *R. solani* but to our knowledge, few reports are available on use of *C. jejuense*, *E. cloacae* and *K. pneumoniae* against this pathogen. Disease suppression achieved using *Chryseobacterium* B12 is in accordance with Krause et al. [44] findings where *C. gleum* was shown to be a putative biocontrol agent able to suppress Rhizoctonia damping-off on several plants.

The current study clearly demonstrated that all treatments performed using the rhizobacterial isolates had significantly increased plant growth parameters i.e. plant height by 62-73%, aerial part fresh weight by 34-67%, and root fresh weight by 53-86%. Thus, these findings showed the additional growth-promoting effects exhibited by the rhizobacterial collection when challenged to tomato plants already infected with the pathogen. According to Ahmad et al. [51], an efficient biocontrol agent is generally equipped with several tools allowing both plant growth promotion and target pathogen inhibition due to their efficient root colonization, phytohormone production ability and nutrient competition. Regarding plant growth-promoting (PGP) properties characterizing this bacterial collection, our previous study demonstrated that 20 isolates among the 25 tested were able to produce siderophore, 18 had solubilized phosphate, 19 were capable to synthesize IAA, and that interestingly 13 isolates showed positive response to at least two PGP traits [23].

Other effects lead to growth promotion during PGPR-plant interactions such as increased root permeability, enhanced ability to survive in strict competitive niche and inhibition of harmful microorganisms [52]. In the present investigation, among the tested rhizobacteria collection, the most promising strains combining both disease suppressive and growth-promoting abilities were *B. subtilis* B10, *B. thuringiensis* B2 and *E. cloacae* B16. Also, interestingly, *C. jejuense* B12 and *K. pneumoniae* B11 isolates had also reduced Rhizoctonia Root Rot severity and augmented root growth and plant height by more 70% compared to pathogen-inoculated control. These tomato-associated agents are able to produce antibiotic lipopeptides, IAA, and siderophore and to solubilize phosphate [23]. *Bacillus* and *Pseudomonas* species are extensively reported as phosphate solubilizing and as IAA- and siderophore-producing agents compared to the other species of rhizobacteria [53]. Additionally, Almaghrabi et al. [54] noted significant increment in shoot dry weight, plant height, and yield in tomato plants treated with *B. amyloliquefaciens*, *B. subtilis* and *B. cereus* and other rhizobacterial species such as *Serratia marcescens*, *Pseudomonas putida*, *P. fluorescens*.

In addition, in the current study, *Enterobacter*, *Chryseobacterium* and *Klebsiella* isolates cumulated the three PGP traits. Also, previous

studies demonstrated that *Chryseobacterium* species represent an important bacterial group associated with plants and displaying interesting plant-growth promoting activities [55-57]. *C. balustinum* pepper-associated rhizobacteria also showed PGP properties and had improved aerial surface, aerial length and the dry weight of the above- and underground plant parts [58]. Contrarily, reports on *Klebsiella* species as PGP agents are relatively rare. In fact, Ahemad and Khan [59] found that *Klebsiella* sp. strain PS19 solubilized the inorganic phosphate considerably, produced IAA and siderophores. *K. oxytoca* Rs-5, isolated from Chinese saline cotton fields are able to attenuate salt stress, to enhance plant growth and to release IAA [60]. Sachdev et al. [61] found that IAA-producing *Klebsiella* strains had significantly improved root length and shoot height of infected wheat seedlings relative to control.

Conclusion

The present investigation clearly demonstrated that tomato rhizospheric soils removed from tomato-growing sites of Tunisia harbor interesting biocontrol candidates belonging to *Bacillus*, *Enterobacter*, *Chryseobacterium*, and *Klebsiella* genera. These tomato-associated rhizobacteria displayed Rhizoctonia Root Rot suppression ability and plant growth promoting capacity. Thereby, they could be developed as biofertilizing and biocontrol agents once their effectiveness demonstrated under field conditions and in different tomato-producing sites. Further studies will be focused on the assessment of their disease-suppressive effects against Rhizoctonia Root Rot disease and their plant growth-promoting potential when used as consortia and the eventual shifts in rhizosphere microbial community occurring after their release.

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