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Characterization of Tomato-associated Rhizobacteria Recovered from Various Tomato-growing Sites in Tunisia

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Abstract

In the present study, a total of 200 rhizobacterial isolates were obtained from rhizosphere of healthy tomato plants grown in fields with a history of severe soilborne diseases and mainly crown and root rots. Screened their capacity to suppress *in vitro* growth of *Sclerotinia sclerotiorum* and *Rhizoctonia solani*, 69 and 57 isolates out of the 200 tested were shown able to inhibit significantly the mycelial growth of target pathogens by 11-62% relative to control. The 25 most effective isolates, leading to suppression of both fungi by more than 45% over control, were selected and subjected to morphological, biochemical, molecular, and metabolic characterizations. This collection of tomato-associated rhizobacteria exhibited a great morphological and biochemical diversity. Sequencing of 16S rRNA and *rpo*B genes led to the identification of four genera namely *Bacillus*, *Chryseobacterium*, *Enterobacter*, and *Klebsiella*. The most frequent species were *B. amyloliquefaciens*, *B. thuringiensis*, *B. megaterium*, *B. subtilis*, *E. cloacae, C. jejuense*, and *K. pneumoniae*. Screening for their plant growth-promoting properties, 20 isolates were shown able to produce siderophore, 18 had solubilized phosphate, and 19 were capable to synthesize indole-3-acetic acid (IAA). PCR amplification of lipopeptide biosynthetic genes revealed the presence of genes encoding fengycin A and bacillomycin D biosynthesis in 18 and 16 isolates, respectively. Metabolic characterization performed using Biolog™ Ecoplates indicated that tomato-associated rhizobacteria displayed a large metabolic activity and they were able to use a wide range of carbon sources with the increase of the incubation duration. Based on their metabolic profiles, these rhizobacterial isolates were grouped into eight major clusters generated at the different sampling times (24, 48 and 120 h of incubation). Average well-color development (AWCD) values were found to be positively correlated with the Shannon diversity index.

Keywords: Antifungal activity; Characterization; Diversity; Identification; Rhizobacteria; Tomato; Tunisia

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown vegetable crop worldwide and is ranked second after potato [1]. In Tunisia, tomato is a strategic crop but unfortunately threatened by various bioagressors and mainly soilborne fungi due to the practice of monocropping and the lack of crop rotation due to the limited producing areas [2-5]. Moreover, the widely grown tomato cultivars were susceptible to soilborne diseases and especially to Rhizoctonia Root Rot caused by *Rhizoctonia solani* and Sclerotinia Stem Rot induced by *Sclerotinia sclerotiorum*. These pathogens are responsible for damping-off and/or collar, stem and roots rots and eventually plant death leading to serious yield losses both under greenhouse and open field growing systems [6,7]. They survive in soil either as sclerotia or as mycelium in crop debris [8]. Currently, few fungicides are effective in controlling these serious pathogens but even when allowed, chemical tools still involve technical, environmental and toxicological risks [9]. Moreover, it is very difficult to protect tomato plants from crown and root rot diseases using fungicides only particularly when the crown zone was covered with dense plant foliage. Such issues have encouraged research efforts on new, effective, and eco-friendly alternative methods such as biocontrol [10].

Several microorganisms have been reported as effective biocontrol agents (BCA) for suppression of plant diseases caused by soilborne pathogens [11-13]. A particular interest was attributed to root-associated bacteria which displayed interesting antagonistic potential towards *Rhizoctonia* spp. [14]. Plant growth promoting rhizobacteria (PGPR) produce various types of antifungal metabolites, including antibiotics and siderophores, capable of reducing or suppressing infection by pathogenic fungi in several pathosystems [15,16]. Furthermore, rhizobacteria have received particular attention because of their excellent root colonizing ability and their ability to produce a wide range of antifungal metabolites and auxins such as indole-3-acetic acid or IAA) [17-19].

Due to the limited knowledge in Tunisia on the nature of native tomato-associated rhizobacteria and their ability to suppress root rot diseases in tomato, the main objectives of the present study were i) to isolate bacterial isolates from tomato rhizosphere and to assess their ability to suppress the *in vitro* growth of *S. sclerotiorum* and *R. solani*, ii) to identify and characterize the most effective ones in suppressing both pathogens, and iii) to assess their potential to produce antifungal and plant growth-promoting compounds.

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Materials and Methods

Soil sampling and processing

Tomato-associated rhizobacteria were isolated from the rhizospheric soils of apparently healthy and vigorous tomato plants grown in various fields and greenhouses with a history of severe soilborne diseases. Surveys concerned different tomato-growing sites namely Kairouan, Sidi Bouzid, Cap-Bon, Sahline and Chott-Mariem regions (Table 1). Soil samples were removed from the top layer (15-20 cm deep) using a small shovel. Each soil sample was placed in a separate plastic bag before being brought to the laboratory and processed.

Bacterial isolation and culture

A subsample of 1 g was taken from each air-dried soil sample and homogenized in 10 mL SDW. The obtained soil suspension was shaken at 150 rpm at 25°C for 24 h. The resulting sludge was filtered and serially diluted four times (10^{-1} to 10^{-4}), and 0.1 ml of each diluted suspension was injected in a separate Petri dish containing 10 ml of molten Levan Peptone Glucose Agar (LPGA) and stirred well before medium solidification.

After 2 to 3 days of incubation at 28°C, distinct individual developing bacterial colonies were selected based on of their macro morphological diversity and picked separately onto Nutrient agar (NA) medium. Purification was performed by streaking out each distinct type of colony on NA in a separate plate and grown during 48 h at 28°C before being used for characterization bioassays. Stock cultures were stored at -20°C in Nutrient Broth (NB) medium supplemented with 15% glycerol.

Assessment of antifungal potential of collected rhizobacteria

R. solani and *S. sclerotiorum* isolates used for the assessment of the antifungal potential of the collected rhizobacterial isolates was originally recovered from naturally infected tomato plants exhibiting typical Rhizoctonia Root Rot and Sclerotinia Stem Rot symptoms, respectively. Fungal cultures were gratefully provided by the Plant Pathology Laboratory at the Regional Centre of Research on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia. They were grown onto Potato Dextrose Agar (PDA) medium supplemented with streptomycin sulfate (300 mg/L w/v) and incubated at 25°C during 5 days before use.

A total of 200 rhizobacterial isolates was collected from the different sampling sites. They were evaluated for their antifungal activity against both target pathogens using the dual culture technique. Agar plugs (5 mm in diameter), removed from 5-day old cultures, were plated at one side of a Petri plate containing PDA. From a 48 h -old culture, 10 µL of bacterial cell suspension (10^8 CFU/mL) were injected into a well (5 mm in diameter) performed at the opposite side using a sterile cork borer. Plates challenged with pathogen plugs only were used as control. Each individual treatment was replicated thrice. The whole experiment was repeated twice.

Geographical location	Growing region	Crop type and number of samples		Cropping system
		Protected	Open field	
Cap Bon	Korba	-	5	Conventional
	Takelsa	5	-	
Centre	Chebika (Kairouan)	-	10	Conventional
	Sidi Ali Ben Salem	-	5	
Centre-Ouest	Regueb (Sidi Bouzid)	-	5	Conventional
Sahel Centre-East	Chott-Mariem	5	-	Organic
	Sahline	5	-	Conventional
	Téboulba	5	-	Conventional
	Ouardanine	5	-	Conventional
	Békalta	5	-	Conventional
	Ouled chamekh	5	-	Conventional
Total	-	**35**	**25**	

Table 1: Origin and number of soil samples used for isolation of tomato-associated rhibacteria.

Plates were incubated at 28°C for 5 days. The diameter of target pathogen colony was measured and the percentage of growth inhibition was calculated using the following formula [20]:

$$GI(\%) = \frac{(C\text{-}T)\times 100}{C}$$

where I: Percent growth inhibition; C: Colony diameter of the target pathogen in control plates; T: Colony diameter of the target pathogen dual cultured with rhizobacterial isolates.

A growth inhibition score (GIS), estimated based on GI values, was attributed to each rhizobacterial isolate tested using an arbitrary 0-5 scale where 0 = no inhibition, 1= GI 1- 15%; 2 = GI 16- 35%; 3 = GI 36-45%; 4 = GI 46-65%, and 5 = GI ≥ 66%.

The most effective 25 isolates in suppressing growth of both pathogens by more than 45% (GIS estimated at 4 and more) will be selected for further investigations.

Characterization of selected rhizobacterial isolates

To explore the diversity of tomato-associated rhizobacteria, the selected 25 bacterial isolates were subjected to various characterizations.

Morphological and biochemical characterization

Morphology of bacterial colonies was characterized based on various traits such shape, elevation, surface, margin, color, and pigmentation. This macro-morphological characterization was performed, following Patel et al. [21] protocol, on bacterial cultures previously grown for 48 h on NA medium and incubated at 28°C.

The biochemical traits considered in the present study were the Gram staining, levan activity, catalase activity, oxidase activity, and ability to produce fluorescent pigment on King's B medium (KB) [22]. Results of these tests were scored as positive or negative.

Characterization for plant growth-promoting traits

Siderophore production: Bacterial isolates were grown on NA medium for 48 h at 28°C. Siderophore production was assessed by spot inoculation of bacterial isolates onto the Chrome Azurol S (CAS) agar medium. Control plates were spot treated with SDW only. Plates were incubated at 28°C for 5 days. Development of yellow-orange halos around the growing colonies indicated positive siderophore production as indicated by Shobha and Kumudini [23]. The halo diameter was measured and compared to the untreated control.

Phosphate solubilization: Phosphate solubilization ability of isolates was assessed qualitatively using a modified Pikovskaya's agar medium containing tricalcium phosphate according to Venkadesaperumal et al. [24] method. Briefly, each bacterial culture was spot inoculated in the centre of a Petri plate and incubated at 28°C for 7 days. Control plates were treated with SDW only. Clear halo zones formed around colonies indicate positive Phosphate solubilization activity.

Indole-3-Acetic Acid (IAA) production: Production of the phytohormone indole-3-acetic acid (IAA) by the selected isolates was assessed using Ghodsalavi et al. [25] protocol. Briefly, each bacterium was grown onto NB and incubated at 28°C for 48 h in an incubator-shaker at 170 rpm. Then, 50 µL of each bacterial liquid culture were transferred to NB containing 50 µg/mL of L-tryptophane. After 48 h of incubation, the bacterial suspensions were centrifuged at 10000 rpm for 10 min. About 1 mL of supernatant was mixed with 4 mM of Salkowski reagent (1 mL 0.5 M $FeCl_3$ and 50 mL of 35% $HClO_4$) [26]. Reagent mixed with SDW was used as control. After 30 min of incubation, the inoculated reagents that turned red indicate that the tested isolate is an IAA-producing agent.

Characterization of the hypersensitive reaction: The tomato-associated rhizobacteria isolated were also screened for their hypersensitive reaction (HR) on tobacco leaves. Each isolate was suspended in SDW and the concentration of the cell suspension was adjusted to 10^8 cells/mL using a heamocytometer. The bacterial suspension was injected into tobacco leaves. Leaves injected with *Pseudomonas syringae* pv. *tomato*, a phytopathogenic bacteria, were used as positive controls and others injected with SDW only were considered as negative control. Bacteria with positive reaction i.e. causing the development of necrotic area 24 h after injection were ranked as pathogenic and therefore excluded from any further investigation on their antagonistic potential as suggested by Nawangsih et al. [27].

Metabolic characterization: Metabolic characterization of the 25 bacterial isolates was performed using the Biolog® system. Among the 31 substrates present in triplicate, 10 contained carbon and nitrogen (including six amino-acids), 2 composed of carbon and phosphorus and 19 with only carbon. Bacterial cells were recuperated after centrifugation at 3000 rpm during 30 min at 15°C. The pellet cells were resuspended in 15 mL of 0.85% NaCl solution. About 150 µL of the obtained bacterial cell suspension were dropped in each well of the Biolog® EcoPlates as indicated in Renault et al. work [28]. The plates were incubated at 28°C in darkness. The rate of substrate use was indicated by the reduction of the tetrazolium, a redox indicator dye turning from colorless to purple. The color development was determined at 590 nm with Microlog-System Release 4.0 software absorbance after 24 h, 48 h and 120 h incubation.

Microbial response in each microplate, expressed through average well-color development (AWCD), was determined according to Gomez et al. [29] using the following formula:

$$AWCD = \sum ODi/31$$

where OD_i is the optical density in each well, corrected by subtracting the water blank well (inoculated but without any carbon source). The reading for each well was then corrected by subtracting the values of the water blank for a given replicate, and standardized by dividing by AWCD for that replicate as indicated in Garland and Mills [30].

If OD_{ijt} represents the corrected optic density for well for replicate j at time t, then the AWCD for replicate j at time t is given as follows:

$$AWCDjt = \frac{1}{31}\sum_{i=1}^{n31} OD_{ijt}$$

The standardized OD values are determined as follows: $\overline{ODijt} = \frac{ODijt}{AWCDjt}$.

The Shannon diversity index (H), which was commonly used to characterize species diversity within a microbial community [8,30], was calculated to assess the diversity of the 25 rhizobacterial isolates using the following Chojniak et al. [31] formula:

$$H = -\sum(P_i \times \ln P_i)$$

where P_i is the proportional optical density value of each well.

The relationships among different samples on the basis of the transformed data were determined using Principal Component Analysis (PCA) according to Grove et al. [32]. This technique allowed projection of the original data onto new axis (principal components) thus, reflecting any intrinsic pattern in the multidimensional data group [30].

Molecular identification and detection of antibiotic producing genes

DNA was extracted from pure cultures grown in NB using the Invisorb Spin Plant Mini Kit (Invitek) according to manufacturer's instructions. The DNA extracts were then quantified with a nanodrop (ND-1000, Thermoscientific, Labtech) and homogenized at a concentration of 10 ng/µL.

Bacterial isolates were first identified by 16S rDNA sequence analysis, amplified with the universal primers 799f/1492r. For distinction of isolates belonging to *Bacillus* genus, two other genes were sequenced i.e. *rpo*B and *gyr*B. Also, all isolates were screened for 5 antibiotic-producing genes i.e. 4 fengycins (A, B, D, and E), and one gene encoding bacillomycin D biosynthesis (Table 2).

The 8 genes were amplified under the same PCR conditions. Briefly, each PCR mixture contained 2 µL of extracted DNA (10 ng/µL), 1 µL of $MgCl_2$ (50 mM), 0.6 µL of dNTP (10 mM), 0.6 µL of each primer (10 ng/µL), 3 µL Bovine Serum Albumine (10 µg/µL) (BioLabs), 0.1 µL of Taq polymerase (SilverStar DNA polymerase, Eurogentec), 3 µL of PCR buffer (10X) and 19.7 µL of SDW in a total volume of 30 µL.

PCR amplification reactions were performed in a Mastercycler Gradient thermocycler (Eppendorf) using De Clerck and De Vos method [33]. The following cycling conditions were used: initial

Primer name	Sequence	Amplicon size (pb)	Annealing Temperature (°C)
799f	AACMGGATTAGATACCCKG	400	52
1492r	GTTACCTTGTTACGACTT	400	52
rpoB-f	GACGATCATYTWGGAAACCG	500	52
rpoB-r	GGNGTYTCRATYGGACACT	500	52
gyrB-f	AAAACAACCRATTCATGAAG	500	52
gyrB-r	TCGCTTCACTATTYCCAAGT	500	52
FENA1F	GACAGTGCTGCCTGATGAAA	757	54
FENA1R	GTCGGTGCATGAAATGTACG		
FENB1F	CAGCCGCTGTCAACAAGATA	950	54
FENB1R	ACACGACATTGCGATTGGTA		
FEND1F	TTTGGCAGCAGGAGAAGTTT	964	53
FEND1R	GCTGTCCGTTCTGCTTTTTC		
FENE1F	GCCAAAAAGAAACGAGCAAG	756	53
FENE1R	GTCGGAGCTAACGCTGAAAC		
BACC1F	GAAGGACACGGCAGAGAGTC	814	60
BACC1R	CGCTGATGACTGTTCATGCT		

Table 2: Pairs of primers used for identification of tomato-associated rhizobacteria and detection of antibiotic biosynthesis genes.

denaturation of 5 min at 94°C followed by 30 cycles of denaturation (1 min at 94°C), annealing of primers (45 s at 52°C), elongation (1 min at 72°C) and a final elongation for 8 min at 72°C. Amplified fragments were purified and sequenced by GATC Biotech (Germany). For species level identification, sequences were compared with the Genbank database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [34]. Then, sequences were deposited in the Genbank database for getting the accession numbers.

Statistical analysis

Mean colony diameters of each target pathogen depending on bacterial treatments tested (200 isolates and untreated control) were analyzed using one-way analysis of variance according to a completely randomized design. Means were separated using the Duncan's Multiple Range test at ($P \leq 0.05$). Based on growth inhibition records relative to control, isolates were ranked into different classes as previously described and the number of isolates within each class was determined. Data analyses were performed using SPSS version 16.0 and each individual treatment was replicated thrice.

Results

Assessment of the antifungal potential of tomato-associated rhizobacteria towards *Rhizoctonia solani* and *Sclerotinia sclerotiorum*

All the 200 bacterial isolates collected were screened for their antifungal potential against two tomato fungal pathogens namely *S. sclerotiorum* and *R. solani* using the dual culture method. Their effects on pathogen colony diameter were compared to the untreated control. ANOVA analysis, performed for each target pathogen, revealed that mean colony diameter, noted after 5 days of incubation at 25°C, varied significantly (at $P \leq 0.05$) depending on bacterial treatments tested (collected isolates and control). In fact, 69 isolates out of the 200 tested had significantly inhibited by 11.11 to 62.22% the mycelial growth of *S. sclerotiorum* relative to the untreated control (data not shown). Based on growth inhibition scores (GISs) exhibited toward *S. sclerotiorum*, 155 isolates were ranked within class 1 indicating that their relative percentages of growth inhibition were less than 15%. However, interestingly, 27 isolates led to more than 45% decrease in pathogen growth and were, thus, ranked within class 4 (Figure 1).

As for *R. solani*, mycelial growth was significantly lowered by 11.85 to 61.48%, compared to the untreated control, when dual cultured with 57 isolates. Data shown in Figure 1 indicated that when assessed toward *R. solani*, 151 isolates had inhibited by less than 15% pathogen growth versus control and were thereafter ranked within class 1 but, interestingly, more than 45% decrease in pathogen radial growth was achieved using 36 isolates.

The most effective 25 rhizobacterial isolates against both targeted pathogens and raked with class 4 were selected for further identification and characterizations.

Morphological and biochemical characterization of tomato-associated rhizobacteria

Based on their macro-morphological traits on NA medium, the selected isolates exhibited a great diversity color in their color (white, transparent, cream, light yellow), shape and texture as shown in Table 3.

The results of biochemical characterizations indicated also a large diversity in their biochemical traits. In fact, among the collected rhizobacteria, 18 isolates out of the 25 tested were Gram positive and the remaining ones were Gram negative. The oxidase activity (oxidase +) was detected in 15 isolates and 2 were oxidase +/-. The majority of isolates (22/25) were Levane – and 3 were Levan +/-. Catalase positive activity (catalase +) was detected in 17 isolates while 5 exhibited catalase +/- and the remaining isolates gave negative reaction. It should be also mentioned that all the isolates tested did not produce fluorescent pigments in King's medium B; this result indicated the absence of *Pseudomonas fluorescens* within the bacterial collection subjected to this chemical characterization (Table 3).

Molecular identification of tomato-associated rhizobacteria

In the present study, molecular identification of 25 isolates was performed using the 16S rRNA and *rpo*B gene sequences. Based on 16S rRNA gene sequencing, the results revealed that the collected isolates belonged to different genera; the sequence analysis of 16S rRNA (alignment on 700 pb) obtained for *Bacillus* sp. was similar to each other and did not allow evident distinction at the species level although the fragments revealed more than 98% similarity. In fact, as shown in Table 3, among the 25 isolates tested, 20 were identified as

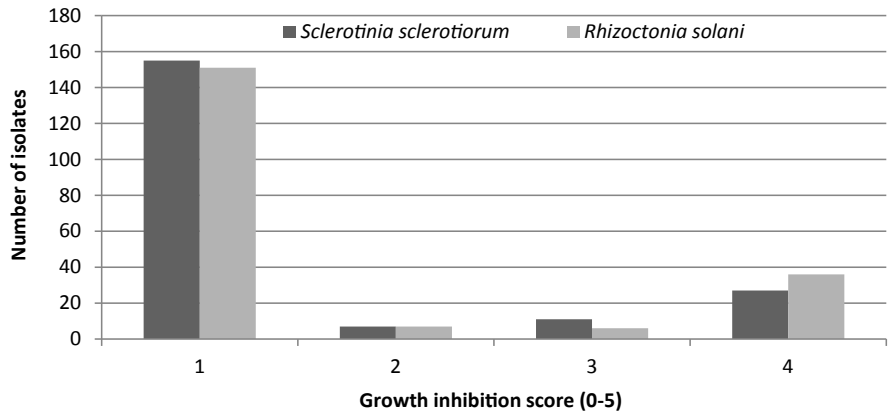

Figure 1: Number of tomato-associated rhizobacterial isolates and their repartition into four growth inhibition classes depending on their relative ability to suppress the *in vitro* growth of *Sclerotinia sclerotiorum* and *Rhizoctonia solani* using the dual culture method. Growth inhibition score was estimated based on growth inhibition (GI) percentage calculated using Prashar et al. [20] formula using an arbitrary 0-5 scale where 0 = no inhibition, 1= GI 1- 15%; 2 = GI 16- 35%; 3 = GI 36-45%; 4 = GI 46-65%, and 5 = GI ≥ 66%.

Molecular identification			Morphological characterization	Biochemical characterization				Detection of antibiotic biosynthesis genes		PGPR traits		
Isolate	Similarity (%)	Accession No.	Colony morphology	Gram	Oxidase	Catalase	Levan	Fen A	Bac D	IAA	P. Solubilization	Siderophore production (halo diameter in mm)*
Bacillus megaterium B1	99	KU168423	White, flat, opaque	-	+	+	-	-	+	+	-	48.3
B. thuriengiensis B2	99	KU158884	White, flat, opaque	+	+	+	-	-	+	+	+	31.7
Enterobacter cloacae B3	99	KT923049	White, flat, opaque	-	-	+	-	+	-	+	+	55.0
E.cloacae B4	100	KT923050	Transparent, flat, Translucide	-	-	+	+/-	+	+	+	+	50.0
B. megaterium B5	100	KT923054	Transparent, flat,Translucide	-	+/-	+	+/-	+	-	+	+	45.0
B. subtillis B6	100	KT921427	White, convex, translucide	+	-	+	-	+	+	-	+	0.0
B. amyloliquefaciens B7	99	KT921428	White, flat, opaque	+	-	+	-	+	+	+	-	83.3
B. subtillis B8	99	KU158885	White, flat, opaque	+	+	+	+/-	+	+	+	+	35.0
B. amyloliquefaciens B9	100	KU158887	Transparent, convex, opaque	+	+	-	-	+	+	+	+	36.7
B. subtillis B10	99	KT921327	Light yellow, flat, opaque	+	+	+	-	+	+	+	+	90.0
Chryseobacterium jejuense B11	99	KU158886	White, convex, translucide	+	-	-	-	-	+	+	+	43.3
Klebsiella pneumoniae B12	99	KT921328	Transparent, convex, translucide	-	-	+	-	+	-	+	+	90.0
B. amyloliquefaciens B13	99	KT951658	Transparent, convex, translucide	+	+	+/-	-	+	-	+	+	36.7
B. subtillis B14	99	KU161090	White, flat, opaque	+	+	+/-	-	+	-	-	-	73.3
B. amyloliquefaciens B15	99	KT923051	White, plate, opaque	+	+	+/-	-	-	+	-	-	28.3
E. cloacae B16	100	KT921429	Cream, flat, opaque	-	-	+	-	+	+	+	+	90.0
B. subtillis B17	100	KT923055	White, flat, opaque	+	-	+	-	+	+	+	+	0.0
B. amyloliquefaciens B18	100	KT923052	White, plate, opaque	+	+	-	-	+	+	+	+	0.0
B. subtillis B19	99	KT921430	Cream flat, opaque	+	+	+/-	-	+	-	-	-	75.0
B. subtillis B20	99	KT921431	White, plate, opaque	+	+	+	-	+	+	+	+	66.7
B. amyloliquefaciens B21	100	KT923047	Transparent, plate, translucide	+	+	+	-	+	+	+	+	0.0
B. amyloliquefaciens B22	100	KT923053	White, plate, opaque	+	+	+	-	+	+	+	+	0.0
B. thuriengiensis B23	99	KT923056	White, plate, opaque	+	+	+	-	+	+	+	+	13.3
B. megaterium B24	100	KT923048	Cream, flat, opaque	-	+	+	-	-	+	-	-	75.0
B. subtillis B25	99	KU161091	Transparent, flat, Translucide	+	+/-	+/-	-	-	+	-	-	10.0

Table 3: Molecular, morphological and biochemical characterization of tomato-associated rhizobacteria and assessment their ability to produce lipopeptide antibiotics and plant growth-promoting compounds. Positive reaction (+); Negative reaction (–); Fen A : Fengycin A, Bac : Bacillomycin D, IAA: Indole-3- 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. *: Average diameter of orange halo of the siderophore production by rhizobacteria. PGPR: Plant growth promoting rhizobacteria.

belonging to the genus *Bacillus*, three as being *Enterobacter cloacae*, one as *Chryseobacterium jejuense* and one as *Klebsiella pneumoniae*.

Unlike the level of sequence similarity noted between *Bacillus* isolates using the 16S rRNA gene sequencing, *rpo*B gene sequences showed more variation and were used to identify isolates at the species level. In fact, the *rpo*B amplicons were of approximately 400 bp in length and the sequence similarity of the collected isolates and the bacterial sequences available at the Gene Bank ranged from 99 to 100%. Based on comparison of the partial sequences using BLAST, 3 isolates (B1, B5 and B24) were identified as *B. megaterium*, 9 isolates (B2, B6, B8, B10, B14, B17, B19, B20, and B25) were affiliated to *B. subtilis*, 7 isolates (B7, B9, B13, B15, B18, B21 and B22) were attributed to *B. amyloliquefaciens*, and one isolate (B23) was determined as being *B. thuriengiensis*.

Sequences of the isolates, used in the present study, were submitted to the GenBank and their relative accession numbers are cited in Table 3.

Detection of antibiotic biosynthesis genes

To determine whether the collected tomato-associated rhizobacteria have the potential to produce different types of antimicrobial lipopeptides, PCR amplifications were performed to

detect genes responsible for fengycin (A, B, D and E) and bacillomycin D biosynthesis. Among the 5 primer pairs tested, 2 genes encoding fengycin A and bacillomycin D biosynthesis were detected. Among the 25 isolates screened using FENA1F/FENA1R primer pairs, 18 yielded the indicative presence of *fen*A gene involved in fengycin A biosynthesis. Screened using BACC1F/BACC1R primer pairs, 16 isolates were found to be bacillomycin D-producing agents (Table 3 and Figure 2).

After sequencing the antibiotic biosynthesis genes, the results showed that the gene encoding the enzymes of fengycin A production does not distinguish several different haplotypes within the tested collection of isolates and the obtained sequences were aligned on 757 pb. However, the gene encoding bacillomycin D biosynthesis allowed the discrimination of 3 different haplotypes (13 isolates attributed to the same haplotype and 2 having a distinct haplotype, namely B15 (6 mutations) and B22 (10 mutations) (Figure 2).

Data given in Table 3 also indicated that among the rhizobacterial isolates screened for peptide synthetase biosynthetic genes of fengycin A and bacillomycin D, 20 were able to produce at least one of these antibiotics and 15 isolates were shown able to produce both fengycin A and bacillomycin D.

Screening of plant growth-promoting traits

In vitro assays were performed to select bacterial isolates exhibiting interesting plant growth-promoting characteristics. Isolates were screened for siderophore production, IAA production and phosphate solubilization. Data given in Table 3 revealed that 20 out of the 25 isolates naturally associated with tomato plants were found able to produce siderophore on CAS medium agar plates. *B. subtilis* B10 (KT921327), *E. cloacae* B16 (KT921429) and *K. pneumoniae* B12 (KT921328) isolates produced the largest halo on CAS medium agar, reaching 90 mm in diameter, followed by *B. amyloliquefaciens* B7 (KT921428), *B. megaterium* B24 (KT923048), and *C. jejuense* B11 (KU158886) forming halos of 83.33, 75, and 43.33 mm in diameter, respectively.

When screened for their phosphate solubilization ability on PVK agar, 18 isolates out of 25 tested were shown capable to solubilize phosphate as given in Table 3. Also, most of the tested bacterial isolates (19/25) had produced the plant growth-promoting hormone i.e. indole-3-acetic acid (IAA) (Table 3).

It can be concluded through whole analysis of PGPR traits data given in Table 3 that all screened collected bacterial isolates, originally recovered from rhizosphere of apparently healthy and vigorous tomato plants, showed positive response to at least one PGPR trait (siderophore, IAA production or phosphate solubilization) and that interestingly, 13 and 6 strains showed positive result for three and two traits, respectively.

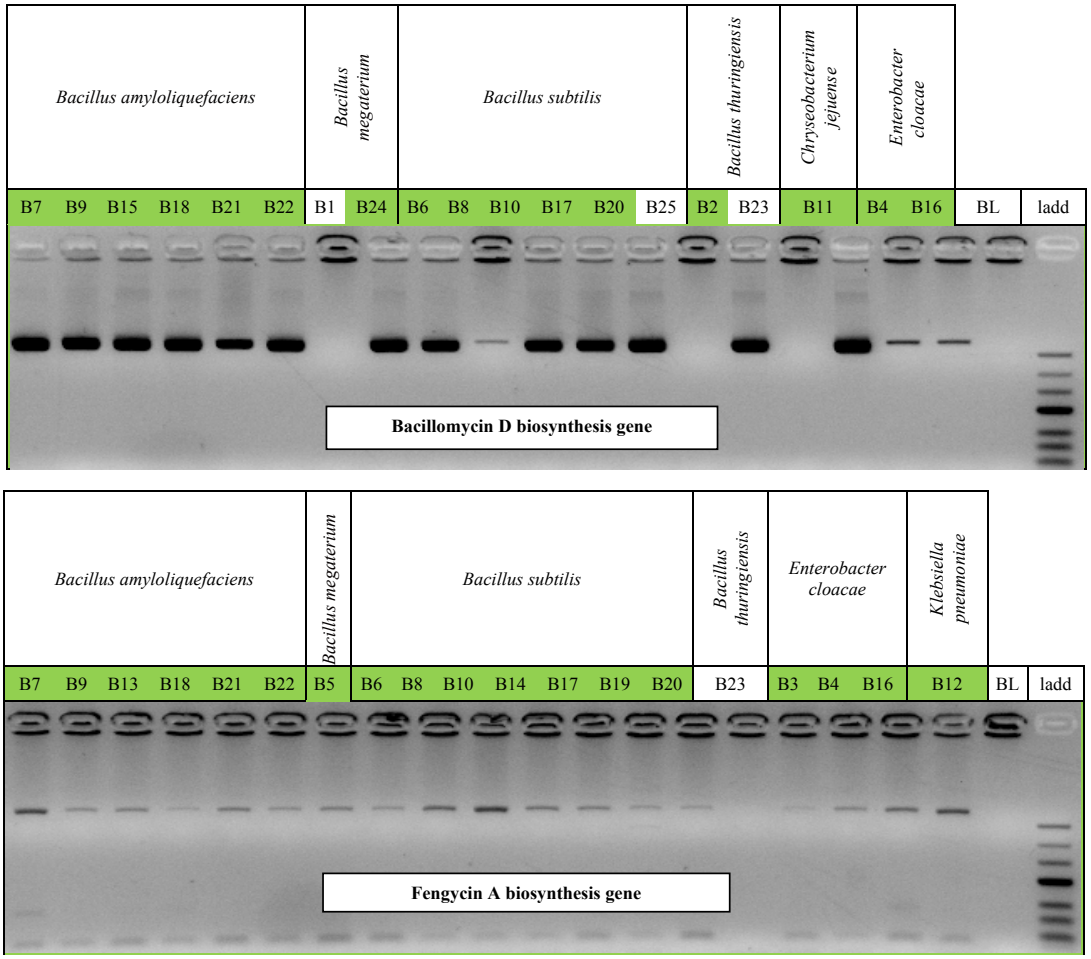

Figure 2: Results of PCR amplification of Bacillomycin D and Fengycin A biosynthesis genes in the 25 tomato-associated rhizobacterial isolates recovered from Tunisian rhizospheric soils. Lane Bl: negative control; lane ladd: molecular weight markers (Ladder).

Hypersensitive reaction on tobacco

Data of this test indicated that no necrotic lesions were observed in all rhizobacteria-inoculated tobacco leaf areas assuming that these 25 bacterial isolates were not pathogenic and were therefore eligible to be used as biocontrol agents once their disease-suppression ability demonstrated in the future.

Metabolic characterization of rhizobacteria collection

The 25 tomato-associated rhizobacterial isolates were assessed for their ability to utilize carbon sources using Biolog™ Ecoplates system based on 31 carbon sources.

The utilization rate was indicated by the reduction of tetrazolium violet, a redox indicator dye that changes from colorless to purple. At the first overall look, all isolates displayed very similar substrate richness indicating a good catabolic activity. Data of the relative metabolic profile of the tested isolates revealed a change in the degree of AWCD with the increase of the incubation duration. Therefore, to more differentiate isolates based on their metabolic profiles and ability to use of the provided carbon sources, a principal components analysis (PCA) was performed.

PCA results given in Figure 3 showed that all isolates were grouped into eight major clusters at each incubation duration (i.e. 24, 48 and 120 h of incubation), indicating that they had different patterns and capacity to metabolize carbon sources. In fact, depending on their metabolic capacity and incubation durations, the bacterial collection tested showed distinctive patterns in their use of carbon source. Figure 3 showed that after 24 h of incubation, the 2 main axes (the 2 first principal components), Dim1 and Dim2, accounted for 65.71% of the total variation. After 48 h of incubation, the two principal components, Dim1 and Dim2, covered 67.32% of the total data variance. After 120 h of incubation, the first and the second principal component (Dim1 and Dim2) explained 51.06 and 9.41% of data variance, respectively, and gave a consistent description of the global database.

The mean scores for each of the 25 isolates tested were calculated and presented to discriminate among microbial populations. Principal components' percentage of the data variance, whatever the duration of incubation considered, clearly separated *B. amyloliquefaciens, B. subtilis* and *C. jejuense* (negative correlation) species from *B. megaterium, E. cloacae* and *K. pneumoniae* (positive correlation). Thus, these isolates exhibited variable capacity to metabolize carbon sources and there was a systematic change in the carbon source use patterns associated with the different genera.

The Shannon diversity index was calculated for each isolate. Data shown in Figure 4 indicated that for all the isolates tested, AWCD values, representing the overall average activity of these bacteria, and their Shannon indexes (calculated for each isolate) were positively correlated ($R^2 = 0.759$).

The average AWCD values recorded at the three incubation durations (24, 48 and 120 h of incubation) ranged between 0.18 and 3.19. The most important values were noted for isolates *E. cloacae* B4 (KT923050) and *C. jejuense* B11 (KU158886). However, average values of Shannon indexes calculated at three reading times varied between 3.2 and 3.40. The highest values were recorded with *E. cloacae* B4 (KT923050) and *B. subtilis* B8 (KU158885) isolates. These results indicate that these native tomato-associated rhizobacteria displayed a large metabolic activity and they were able to use a wide range of carbon sources.

Discussion

Bacterial community in rhizosphere area is known to play a significant effect in protecting plant roots from soilborne pathogens and in improving plant growth [9,35,36]. In the study, bacterial isolates were isolated from soil samples collected around healthy tomato plants grown in different tomato-producing regions of Tunisia. Among a total of 200 isolates collected, 25 were selected based on their ability to suppress by more than 45% *R. solani* and *S. sclerotiorum* growth relative to controls. They were identified and subjected to various characterizations in order to elucidate their main traits involved in fungal biocontrol and eventual plant growth promotion.

For the development of reliable biocontrol agents, it is important to elucidate their mechanisms of action and their nutritional and physiological requirements for optimum growth [37,38]. Thus, deep characterization of a given biocontrol agent is an essential step for improving the level and the reliability of its biocontrol activity [39].

In the present study, the selected 25 isolates were morphologically characterized based on colony color, shape, margin, elevation and

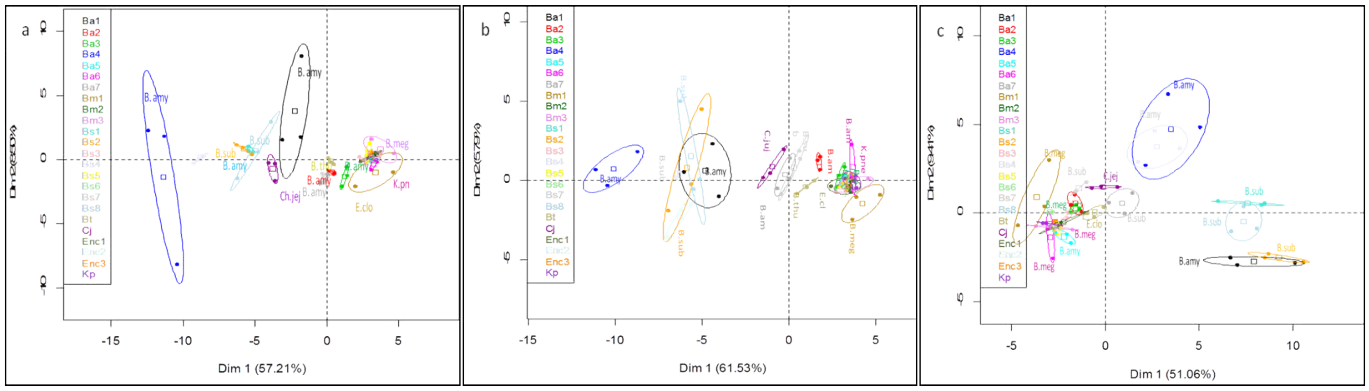

Figure 3: Distribution of the 25 tomato-associated rhizobacterial isolates on the principal plan designed by the global Principal Components (ACP) based on their metabolic profiles using Biolog system based on 31 carbon sources.
a: After 24 h of incubation; b: After 48 h of incubation and c: After 120 h of incubation. For each sample, triplicates are represented by small scores and averaged by a larger one. Percentage of total variation accounted by each PC axis is indicated in parenthesis.
Ba1: B7; Ba2 :B9; Ba3: B13; Ba4:B15; Ba5: B18; Ba6: B21; Ba7: B22; Bm1: B1; Bm2: B5; Bm3: B24; Bs1: B6; Bs2: B8; Bs3: B10; Bs4: B14; Bs5: B17; Bs6: B19; Bs7: B20; Bs8: B25; Bt: B2; Bt: B23; Cj: B11; Enc1: B3; Enc2: B4; Enc3: B16, and Kp: B12.

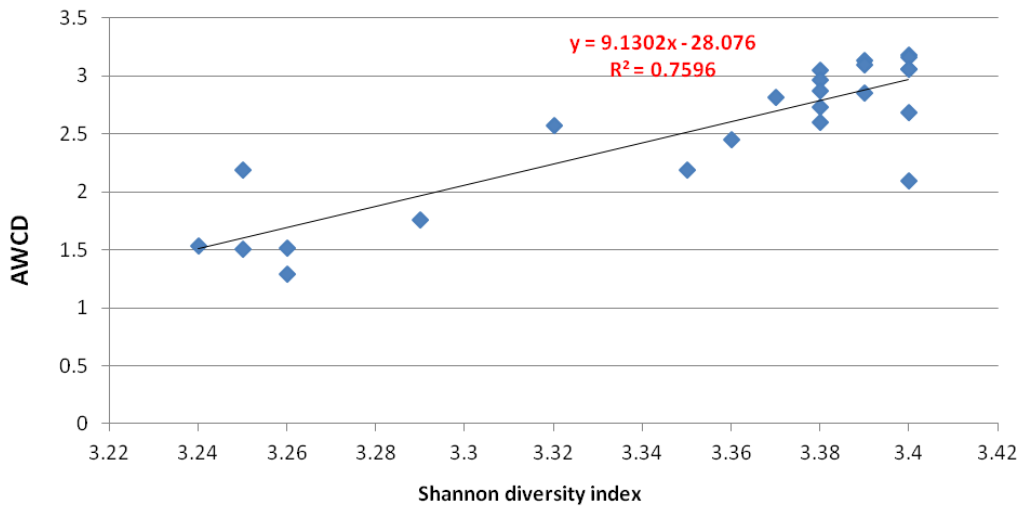

Figure 4: Correlation between average well color development (AWCD) and Shannon diversity index calculated from substrates degraded in Biolog® EcoPlates by the 25 tested tomato-rhizobacterial isolates (performed for means of three incubation durations).

surface where a great morphological diversity was recorded within the bacterial collection tested. Additionally, performed biochemical characterizations revealed the presence of great diversity within tomato-associated rhizobacteria based on Gram staining, levan activity, catalase activity, and oxidase activity.

Identification of biocontrol agents may be performed using biological and molecular methods. The 16S rRNA sequencing is widely used as an alternative method to identify microbial microorganisms. In the present study, the 25 isolates were subjected to 16S rRNA sequences analysis for phylogenetic comparisons. The obtained results revealed that the collected isolates belonged to different genera but, in some cases, the sequences obtained were very similar to each other and did not allow distinction of isolates at the species level especially within *Bacillus* group. Similarly, Chen and Tsen [40] failed in discriminating between species of *Bacillus* using 16S rRNA sequences analysis only. Also, González et al. [41] reported that analysis based on pair wise alignment of 16S rDNA sequences solely showed limited variations and sequence similarities between isolates and strain types were frequently encountered. In the same way, Hutsebaut et al. [42] and 43 Maughan and van der Auwera [43] indicated that the 16S rRNA gene sequences of species within the *Bacillus* group were highly conserved to enable easy and evident discrimination among species and subspecies. In the present study, analysis of 16S rDNA sequences showed limited variation where sequence similarities between isolates ranged between 96.5 and 99%. To overcome this constraint, protein-coding genes are reported to lead to greater genetic variation and were widely used as molecular tool for the classification of closely related species [44-46]. Some genes like *gyr*A and *rpo*B have been proposed to provide conclusive evidence on the evolutionary relationships of related taxa [47,48]. Unlike the high level of 16S rRNA gene sequence similarity noted within *Bacillus* group, sequence of the *rpo*B gene exhibited greater diversity and allowed identification of distinct species [41,49]. The sequencing of the 16S rRNA and *rpo*B genes of the 25 selected isolates revealed a species diversity where the tomato-associated isolates were classified into seven bacterial species namely *B. subtilis* (8 isolates), *B. amyloliquefaciens* (7 isolates), *B. megaterium* (3 isolates), *E. cloacae* (3 isolates), *B. thuringiensis* (2 isolates), *C. jejuense* (1 isolate), and *K. pneumoniae* (1 isolate).

Antibiotic production by bacterial antagonists is an essential component in the biological control of fungal phytopathogens and is characterized genetically through the detection of various antibiotic producing genes as shown in various studies [50-52]. In recent years, cyclic lipopeptide antibiotics have been reported to inhibit a wide range of plant pathogenic fungi [53,54]. In the present study, it was possible to associate the presence of antibiotic in the culture suspension of some strains with their corresponding biosynthetic genes. PCR primers used for the detection of multi-modular enzymes, the peptide synthetases involved in the biosynthesis of antifungal lipopeptides, showed the presence of fengycin A- and bacillomycin D-producing agents within this collection of rhizobacteria. The production of antibiotics by *Bacillus* spp. and their roles in suppression of plant pathogens are mentioned in many reviews [9,55]. *B. subtilis* and *B. thuringiensis* are previously reported to be subtillin, bacitracin, bacillin, subtenolin, and bacillomycin producers [56-58]. The production of cyclic lipopeptides is largely detected within *Bacillus* species but Mandal et al. [59] also demonstrated that *E. cloacae* can also produce different lipopeptide antibiotics like kurstakin, iturin, surfactin, and fengycin.

Chryseobacterium species were also commonly found in soil and water and their ability to suppress some soilborne diseases was reported [60-62]. Their mechanisms of action as biocontrol agents vary depending on species and isolates. In fact, *C. wanjuense* KJ9C8, effective in controlling Phytophthora blight of pepper, produced protease and Hydrogen cyanide (HCN) but not antibiotics [63], and according to Kim et al. [62], ability to produce antibiotics has not been reported in *Chryseobacterium* species. However, in the present study, *C. jejuense* B11 (KU158886) naturally associated to tomato was found to be a bacillomycin D-producing agent. This is, to our knowledge, the first report of presence of bacillomycin D encoding gene in *C. jejuense.* Interestingly, our *K. pneumoniae* B12 (KT921328) isolate was also able to produce fengycin A.

This collection of rhizobacterial isolates was also screened for PGPR properties and particularly siderophore and IAA production and phosphate solublization. In fact, several microorganisms were found to be able to make insoluble soil phosphorous available to plants by solubilizing mineral phosphates through the production of organic acids or phosphatases. In the present study, 18 isolates out of the 25 tested,

belonging to *Bacillus, Enteroabacter, Klebsiella* and *Chrysobacterium,* were shown able to solubilize inorganic forms of phosphorous. *Bacillus* strains were reported to have this capacity [64,65]. The phosphate solubilization ability recorded in some tomato-associated rhizobacteria in the present study is in line with other investigations performed using *Bacillus* [66], *Enterobacter* and *Klebsiella* species [64,67-69].

Siderophore production is another plant growth promoting (PGP) trait that may affect plant growth by binding to the available iron form (Fe3+) in the rhizosphere [70]. Through this process, iron becomes unavailable to plant pathogens; thus plants are protected from biotic stress. In the current investigation, 20 rhizobacterial isolates out the 25 tested were shown able to produce siderophore. Rhizobacteria with siderophore-production ability were reported in various other previous studies as PGP agents [25,71].

IAA is one of the most important phytohormone involved in the regulation of plant growth. IAA production by PGPR can vary between and within species [72]. In the present study, among the 25 isolates tested, belonging to four genera, 19 were found to be IAA producers. Production of phytohormones (e.g. ethylene) by PGPR may affect plant growth and development, and can improve fruit quality as well [73,74].

Overall, based on PGP traits, *Bacillus* and *Pseudomonas* species are well known by their strong phosphorus solubilization activity and by their IAA- and siderophore- production abilities compared to the other rhizobacterial species [75]. Native *Bacillus* spp. isolates, used in the current study, showed PGP traits but also, interestingly, some *Enterobacter*, *Chryseobacterium* and *Klebsiella* isolates cumulated the three traits as they were found capable to produce IAA and siderophore and to solubilize inorganic phosphate. Previous investigations indicated that members of the genus *Chryseobacterium* are an important bacterial group associated with plants [76,77] and exhibiting plant-growth promoting activities [78]. Reports concerning the role of *Klebsiella* as PGPR are relatively rare [79]. Ahemad and Khan [80] demonstrated that *Klebsiella* sp. strain PS19 is able to solubilize inorganic phosphate and produce IAA and siderophores like DHBA (2,3-dihydroxybenzoic acid). Similarly, *K. oxytoca* Rs-5, isolated from a saline cotton field in Xinjiang Province, China, has also the ability to relieve salt stress, promote plant growth and produce IAA [81].

In previous studies, rhizobacteria were reported to have at least on PGP trait. For example, 216 bacterial strains, isolated from rice rhizospheric soils in Northern Thailand, have solubilized inorganic phosphate but only 18.05% of them are able to produce IAA where the best IAA producer was identified as *Klebsiella* SN 1.1 [82]. Also, according to Yasmin et al. [83], two isolates namely *Klebsiella* UPMSP9 and *Erwinia* UPMSP10 produce high IAA with L-tryptophan, solubilize phosphate, produce N in culture, resist to antibiotics but fail to produce siderophore. *K. oxytoca* is able to produce IAA, to solubilize phosphate and displays nitrogenase activity but six *K. pneumoniae* strains, recovered from wheat (var. Lokwan) rhizosphere produce only IAA [79].

Ability to use a sole carbon source, through the Biolog™ EcoPlate test, was widely explored as a reliable method for characterization of microbial communities based on their metabolic profiles [84]. Biolog system based physiological profiling generated a large number of characters and produced robust data and more realistic representation of relationships among strains of a given microbial collection. Data from this study showed that isolates, with similar profiles and being closest, were grouped together. Dawson et al. [85] reported that the metabolic profiling analysis allowed samples to be represented on scatter plots of two or more axes. This analysis is considered to be a highly effective method for differentiating among and within species groups through cluster analysis. In the current study, variable metabolic profiles were found within the rhizobacteria collection tested as indicated by the variations recorded in AWCD values and Shannon indexes. After direct incubation of rhizobacteria isolates in Biolog EcoPlates, results revealed a great diversity as determined through the well color change to violet, permitting colorimetric determination of respiration increase occurring when bacterial cells are oxidizing a carbon source. Liao and Xie [86] indicated that color development seems to mainly reflect species metabolic activity and the ability of a given bacterial community to utilize substrates. Campbell et al. [87] showed that, although the use of environmentally relevant substrates to be beneficial, these substrates would only be useful if their use resulted in the differentiation between samples. Indeed, it can be concluded from the current the study the metabolic profiles may be a useful tool for classifying the soil microbial communities on ecological basis using their functional characteristics (single-use of carbon sources).

Conclusion

The present study was undertaken in order to isolate and identify tomato-associated rhizobacteria from various tomato-producing sites and growing systems and to perform morphological, biochemical, metabolic and molecular characterization for the 25 most effective ones in suppressing growth of *S. sclerotiorum* and *R. solani* by more than 45%.

The current investigation provided strong evidence that tomato rhizosphere was populated by a numerous and a diverse array of rhizobacteria exhibiting a great morphological and biochemical diversity. These 25 isolates were classified into seven species, based on sequencing of 16S rRNA and *rpo*B genes, which were *B. amyloliquefaciens*, *B. megaterium*, *B. subtilis*, *E. cloacae, C. jejuense*, and *K. pneumoniae.* These isolates exhibited a large metabolic activity and were shown able to metabolize a wide range of carbon sources with the increase of the incubation duration. Furthermore, based on their metabolic profiles obtained using Biolog system, the selected isolates were grouped into eight major clusters at each incubation duration (24, 48 and 120 h of incubation) and their AWCD was found be correlated to Shannon diversity index.

Screening of their ability to synthesize plant growth-promoting and antifungal compounds revealed that the majority of isolates were able to produce siderophore, to solubilize phosphate and to synthesize IAA and that interestingly, some isolates cumulated the three plant growth promoting traits. Moreover, when screened for their potential to produce lipopeptide antibiotics, PCR amplification of genes encoding fengycin A and bacillomycin D biosynthesis revealed the presence of these genes in 18 and 16 isolates, respectively. Thus, this study clearly demonstrated that tomato rhizospheric soils harbor a diversity of beneficial bacteria which may be promising biocontrol agents due to their interesting metabolic activity, their capacity to produce lipopeptide antibiotics and their interesting antifungal potential displayed toward both target fungi. Moreover, their ability to produce plant growth-promoting compounds indicated that they may exhibit a bio-fertilizing action. Future investigations will be focused on their ability to suppress root and crown rots caused by *R. solani* and to enhance growth of tomato plants.

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