Biologia (2019) 74:237–250
https://doi.org/10.2478/s11756-018-00169-9

ORIGINAL ARTICLE

Biological control of growth promoting rhizobacteria against verticillium wilt of pepper plant

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Received: 28 March 2018 / Accepted: 22 November 2018 / Published online: 28 November 2018

Abstract

Verticillium dahliae is one of the most important soil pathogens, causing verticillium wilt. It is well known that the use of chemical products against this pathogen is not without side effects on the environment. In this regard, the present study was aimed to search for antagonistic rhizobacteria as an alternative of biological control against this causal agent. A total of 162 isolates were screened for their antagonistic activity according to the "double layer" and the "well diffusion" methods. Three of them (RS11, SF82 and ZO4), were subsequently selected as biological control agent (BCAs) according to their efficiency and were identified by 16S rRNA sequencing and Biolog microplate GEN III as *Bacillus* spp. Using 10 different lipopeptide gene primers, PCR reactions only revealed the involvement of genes responsible for iturins (*ituA, ituD, ituC*), bacillomycin (*bmyA*) and Bacilysin (*bac*A / *B-F, bac*A / *B-R*) biosynthesis. The Plant Growth Promoting Rhizobacteria traits [enzymatic activities, phytohormones production] of the three BCAs were also studied in vitro then on pepper plant (*Capsicum annuum L.*), indicating that *Bacillus subtilis* ZO4 was the most effective, enhancing leaf, stem and root growth comparing to the control.

Keywords *Bacillus* spp. · *Verticillium dahliae* · BCA · PGPR · Iturin

Introduction

Verticillium dahliae is a soil borne plant pathogen that causes vascular wilt in over 160 agronomically important plant species worldwide. Verticillium wilt causes severe economic losses in many crops, including vegetables, fruits, flowers, oilseed crops, fiber crops and woody perennials (Usami et al. 2002; Wei et al. 2015; Markakis et al. 2016). The extraordinary impact of this fungus is largely due to the production of survival structures called microsclerotia, which can persist under field conditions for long periods even in the absence of a host. When the microsclerotia are close to the host plant root and the environmental conditions are favorable, they can easily germinate causing infection (Novo et al. 2017). In Mediterranean countries like Spain (Goicoechea et al. 2001) and Marocco (Douira et al. 2008) wilt is considered one of the most common diseases affecting pepper (*Capsicum annuum* L.).

Control management strategies of *Verticillium dahliae* have been mainly relied on chemical use, such as the antifungal antibiotic aureofungin, the fungicide benomyl, and the plant defense activator acibenzolar-S-methyl (Goicoechea 2009). However, studies have reported ineffectiveness as well as an increasing evidence of fungal resistance to these plant antibiotics (Calderon et al. 2015a, b). On the other hand, promising experimental data have shown that biological control, using selected biological control agents (BCA) could be an alternative approach (Angelopoulou et al. 2014) due to their ability to colonize the rhizosphere and to produce various inhibitory substances (Bhattacharyya and Jha 2012). For instance, rhizospheric bacteria, such as *Pseudomonas fluorescens* have been reported to be effective against the verticillium wilt (López-Escudero and Mercado 2011).

Bacillus species are well known for their ability to control plant diseases through various mechanisms, including the production of several lipopeptides such as bacillomycin,

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mycosubtilin, surfactin, iturin, and fengycin (Fernandes et al. 2007; Snook et al. 2009; Sriram et al. 2011). Among the species, *Bacilus subtilis*, *Bacillus cereus* and *Bacillus amyloliquefaciens* have been particularly reported to be effective for the control of plant diseases caused by soil borne, foliar, and postharvest fungal pathogens (Abeysinghe 2009) in laboratory, greenhouse, and field studies (Soares et al. 2016). Moreover, *Bacillus* spp. are also considered as plant growth promoting bacteria (PGPB), which colonize the root systems of plants and can stimulate plant growth through direct ways including biofertilization, phosphorus solubilization, production of plant hormones (Bent et al. 2001; Reyes et al. 2002), and excretion of diverse compounds like hydrolytic enzymes (Shakeel et al. 2015). Mechanisms of PGPR agents also include reducing the level of disease, induction of systemic resistance, and competition for nutrients and niches (Lugtenberg and Kamilova 2009), without conferring pathogenicity (Van Loon and Bakker 2005; Adesemoye and Kloepper 2009).

The objectives of the present study were first to isolate bacterial strains from a Tunisian Oasis soil and to characterize them based on their morphological, biochemical and molecular attributes; then to study their ability to suppress verticillium wilt disease in pepper plants and finally to assess their plant growth promoting effects.

Materials and methods

Bacterial isolation and culture conditions

For bacterial isolation, rhizosperic soil of the halophytic plant *Zygophyllum album* from an Oasis located in Zarzis, Tunisia was considered. 50 g of soil was sampled, transported and handled on the same day to the laboratory. Suspensions were made by adding 5 g of soil to 50 ml of distilled water and shaking at 200 rpm for 30 min at laboratory temperature. Ten-fold serial dilutions were prepared and one hundred microliters of the 10^{-3} dilution were plated on 25 ml of both Luria-Bertani (LB) medium (Sigma Aldrich). All plates were then incubated for 7 days at 28 °C (Park et al. 2005). A total of 162 isolates were then selected, purified and maintained for long-term storage at −80 °C using glycerol 30%.

Antifungal activity screening and BCAs selection

Bacterial isolates were screened for their ability to suppress the mycelial growth of six (6) fungal pathogens: *Verticillium dahliae* (V4) and *Neofusicoccum mediterraneum* (B0071), both kindly provided to us by the Plant Pathology Laboratory of the University of Rabanales, Cordoba, Spain; and *Fusarium pseudograminearum* (FO1), *Phytophthora* sp. and *Botryosphaeria dothidea* (IOT B002), which were provided by the Labaratory of Improvement and protection of olive genetic resources, Olive Tree Institute of Tunisia.

Dual culture assay A loopful of each of the bacterial isolates was streaked on a fresh Potato Dextrose Agar (PDA) (Sigma Aldrich) plate at approximately 2 cm far from pathogen mycelium plugs. Petri dishes were incubated for 7 days at 24 ± 2 °C. Antagonistic activity was then evaluated by measuring the percentage of growth inhibition (Lahlali and Hijri 2010) according to the formula: $GI(\%) = \frac{R1-R2}{R1} * 100$, where R1 is the distance of fungal growth from the point of inoculation to the colony on control plates (bacteria free), and R2, the distance of fungal growth from the point of inoculation to the direction of the antagonist.

Antifungal activity of cell-free supernatant The three most effective isolates were selected and antifungal activity of their cell-free supernatant (CFS) was evaluated by the well diffusion method. Five ml of soft agar (0.7%) containing a suspension (10^5 conidia ml^{-1}) of each pathogen were poured into PDA plates. After cooling, wells of 6 mm diameter were cut. BCAs suspensions (10^8CFU ml^{-1}) were centrifuged for 15 min, at 8000×g, then neutralized to pH 6.5 and filtered through a 0.22 μm filter. One hundred microlitres of each of the resulting CFS were then placed in the prepared wells. Petri dishes were incubated for 48 h at 24 ± 2 °C and the diameters of inhibition zone were measured according to Ouzari et al. (2008).

Phenotypic and biochemical characterization of BCAs

Phenotypic analyses of selected BCAs was performed using 96-well BIOLOG GENIII Microplates ™ using Omnilog Data collection software (Biolog, Inc., Hayward, CA) (Bochner et al. 2001; Bochner 2003). The test panel was comprised of sources of carbon, nitrogen, amino acids and organics acids; in addition to identifying other important physiological properties such as pH, NaCl and lactic acid tolerance, and susceptibility to antibiotics. Testing for Gram negative staining, oxidase and catalase activities were also performed.

Molecular identification of BCAs

Genomic DNA was extracted from selected BCAs according to Chen et al. (2016), and quantified by spectrophotometry (NanoDrop, Wilmington, USA). The Molecular identification was based on the 16S rDNA gene amplification using the universal primer pairs Fd1 and Rd1 (Issar et al. 2012).

The PCR mix consisted of a final volume of 25 μl containing 100 ng of genomic DNA, 1X My Taq Reaction buffer, 10 μM of each primer, 10 mM dNTP, and 1 U of My Taq DNA polymerase. Amplification was performed with initial denaturation at 94 °C for 3 min, 35 cycles at 94 °C for 1 min,

57 °C for 1 min, and 72 °C for 1 min 30 s, and a final elongation step of 5 min at 72 °C. PCR products were separated using electrophoresis in 1% agarose gel in 1 mol l^{-1} TBE buffer and purified using an Ultra Clean PCR Clean-Up Kit (Mobio Laboratories Carlsbad, CA). The obtained amplicons were sequenced in both forward and reverse directions using an automated sequencer ABI PRISM 3130xl (Applied Biosystems, Foster City, CA) by the division of sequencing of the center of Biotechnology of Borj Cedria, Tunis, Tunisia (CBBC). The nucleotide sequences were edited using BioEdit Sequence Alignment and compared with sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/).

Screening for growth promoting traits and hydrolytic enzymes

Production of indole acetic acid (IAA), ammonia (NH_3) and hydrogen cyanide (HCN)

IAA production was evaluated according to a modified method described by Upadhyay et al. (2009). Bacterial cultures were grown for 72 h in 5 ml of sterile peptone yeast extract broth (peptone – 10 g, beef extract – 3 g, NaCl – 5 g, l-tryptophan – 0.204 g, distilled water– 1 l; pH – 7) and incubated for 96 h in the dark at 28 °C. A volume of 1.5 ml of this broth was then centrifuged for 10 min at 12,850 x g, and 1 ml of Salkawaski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution) was added to 1 ml of the supernatant. The tubes were subsequently incubated for 1 h at 30 °C in the dark and IAA production was indicated by the formation of red color in the medium. Three replicates were considered.

Quantitative analysis was evaluated at 535 nm absorbance using a pf UV/Visible spectrophotometer and concentration was measured according to a standard graph of IAA (HiMedia) obtained in the range of 10–100 μg/ml.

The production of NH3 was evaluated according to Dweipayan et al. (2014). Briefly, 50 μl of bacterial cell suspension was inoculated in 30 ml of peptone broth (4%), then incubated at 28 °C for 72 h, and amended with 1 ml Nessler's reagent. The formation of yellow to brown precipitate indicated a positive reaction. Three replicates were considered.

BCAs were also screened for the production of hydrogen cyanide (HCN) using the method described by Dweipayan et al. (2014). Isolates were streaked on LB medium amended with 4.4 g glycine/l. A Whatman filter paper no.1 soaked in 0.5% picric acid solution (in 2% sodium carbonate) was placed at the top of each plate. Petri dishes (three replicates per BCA) were then incubated at 28 °C for 4 days. The development of orange to red color indicated HCN production.

Phosphate solubilization was evaluated according to (Thabti et al. 2016). A loopful of each BCA was spot-inoculated on Pikovskaya's agar medium (Dinesh et al. 2013). All plates were then incubated at 28 °C for 5 days. The observation of clear zone around the bacterial colony indicated a positive reaction.

Hydrolytic enzymes production

(i). Amylase activity was performed according to Teodoro and Martins (2000). BCAs were cultured on agar starch medium containing 1% soluble starch, 0.2% yeast extract, 0.5% peptone, 0.05% $MgSO_4$, 0.05% NaCl, 0.015% $CaCl_2$ and 2% agar at pH 7.0. All petri dishes were incubated at 28 °C for 48 h. Amylase activity was confirmed by the appearance of a clear halo after staining with Lugol.

(ii). Protease activity was evaluated according to Benkiara et al. (2013). BCAs were spot-cultured on milk agar medium (0.5% tryptone, 0.3% yeast extract, 1.5% agar and 25% skimmed milk), and incubated at 28 °C for 48 h. Protease activity was confirmed by the appearance of a clear zone around the colony indicating the degradation of milk casein.

(iii). Cellulase activity was performed according to Kasana et al. (2008), on CMC agar containing 0.2% carboxymethyl cellulose, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.02% peptone, and 2% agar. Cellulase activity was confirmed by the appearance of a clear halo around the tested BCA after treatment with Gram's iodine. Three plates were used as replicates for each isolate.

(iv). Mannanase activity was evaluated on the LBG medium. After 24 h incubation at 28 °C, all plates were treated with Congo-Red; mannanase activity was identified by the appearance of a clear halo around the tested isolate (Yin et al. 2012).

(v). Urease activity was performed according to Singh et al. (2017). BCAs were cultured on urea indole medium and incubated at 28 °C for 24 h. A color change from yellow to bright pinkish-red indicated a positive reaction.

Identification of antifungal metabolites (AFS)

Physical and chemical properties of antifungal substances

The stability of the antifungal substances was tested against 7 different enzymes: trypsin,α-chymotrypsin, pepsin, peptidase, lysozyme, and proteinase K. Samples of aliquots of CFS were treated with each enzyme at a final concentration of 1 mg ml^{-1}. The test tubes with and without the enzyme (control) were then incubated at 37 °C for 2 h and heated at 100 °C for 3 min in order to denature the enzyme (Compaoré et al. 2013). Thermal stability of the antifungal substances was also analyzed by exposing the CFS to temperatures of 50, 60,

70, 80, 90, and 100 °C for 30 min, as well as an autoclaving at 121 °C for 15 min. Using 1 mol l^{-1} NaOH or HCl, the effect of pH on the antifungal activity was tested by adjusting CFS to pH's of 4, 6, 8, 9 and 10. CFS with adjusted pH, were then incubated for 2 h at 37 °C before being neutralized to pH 7. After each treatment, residual activity was determined using the agar well diffusion method according to the formula: RA $(\%) = (\frac{D}{d}) * 100$; where D: Diameter of maximum activity and d: diameter of inhibition growth.. An untreated CFS was considered as controls.

Detection of lipopeptide synthesis genes by PCR

Polymerase chain reactions were carried out to determine the presence of the iturin, iturin A, mycosubtilin, surfactin, fengycin, bacilysin and bacillomycin biosynthesis genes in the DNA of ZO4, RS11, and SF82 isolates (Table 1). The PCR amplifications were carried out in a 25 µl reaction mixture containing 2 × PCR Master Mix / Dream Taq Green (12.5 µl) (Fermentas GmbH, St Leon-Rot, Germany), high purity sterile water (9.5 µl), 1 µl of each Forward and Reverse primer (10 µmol µl-1) and 1 µl of template DNA. The amplifications were performed in a thermocycler (Applied Biosystems, 2720, Singapore) using the following PCR conditions: 94 °C for 4 min, 30 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 75 s. The final elongation was at 72 °C for 7 min. The amplified products were detected by agarose gel electrophoresis. PCR was positive when a band of the appropriate size was visualized (Compaoré et al. 2013).

In planta experiments

(i) *Pathogenicity test*

Pathogenicity of an isolate of *V. dahliae* was tested on 4 month years old pepper plants. Inoculum was prepared from 1-week old PDB (Potato Dextrose Broth) (Sigma Aldrich) cultures, which was filtrated then diluted at a concentration of 10^6 conidia ml^{-1} (Rekanovic et al. 2007). Inoculation was performed using the root-dip method by soaking for one hour in the prepared suspensions or in sterile distilled water (negative control). Inoculated and control plants were then repotted and incubated for two months at 23 ± 2 °C and 95% humidity, and visible changes were recorded daily.

(ii) *Suppression of Verticillium wilt by BCAs and disease assessment*

Young pepper plants at four months years old were first inoculated as described previously, then treated by watering with either BCA's suspensions (10^8CFU/g of soil) or sterile distilled water (negative control). All plants were subsequently placed at 23 ± 2 °C and 95% humidity for 8 weeks. Three replicates and 15 plants per replicate were considered for each treatment. Disease severity ratings were recorded each two weeks, according to a 0–4 rating scale: (**0** for a healthy plant, **1** = 1 to 33% of a defoliated plant, **2** = 34 to 66% of a defoliated plant, **3** = 67 to 99% of a defoliated plant and **4** = a dead plant) (Trapero et al. 2013). For each replication a disease index (*DI*) was first calculated according to the formula: $DI = (\sum ni * i)/N$: where *i* is the severity (0 to 4), *ni* is the

Table 1 PCR detection of lipopeptide biosynthesis genes for ZO4, RS11 and SF82 isolates

Lipopeptides	Genes	Primers	Primers sequences (5′-3′)	PCR product size expected/ detected	References
Iturin	*ItuD*	ITUD-F1	TTGAAYGTCAGYGCSCCTTT	482 bp/yes	Chung et al. (2008)
		ITUD-R1	TGCGMAAATAATGGSGTCGT		
	ItuC	ITUC-F1	CCCCCTCGGTCAAGTGAATA	594 bp/yes	Chung et al. (2008)
		ITUC-R1	TTGGTTAAGCCCTGATGCTC		
Iturin A	*ituA*	ITUD1F	GATGCGATCTCCTTGGATGT	647 bp/yes	Sarrangi et al. (2009)
		ITUD1R	ATCGTCATGTGCTGCTTGAG		
Surfactin	*srfA*	SRFA-F1	AGAGCACATTGAGCGTTACAAA	626 bp/no	Chung et al. (2008)
		SRFA-R1	CAGCATCTCGTTCAACTTTCAC		
	sfp	SFP-F1	ATGAAGATTTACGGAATTTA	675 bp/no	Chung et al. (2008)
		SFP-R1	TTATAAAAGCTCTTCGTACG		
	srf/lcg	As1-F	CGCGGMTACCGVATYGAGC	419, 422, 425, 431 /no	Stein (2005)
		Ts2-R	ATBCCTTTBTWDGAATGTCCGCC		
Mycosubtilin	*myc/itu*	Am1-F	CAKCARGTSAAAATYCGMGG	416, 419/no	Stein (2005)
		Tm1-R	CCDASATCAAARAADTTATC		
Fengycin	*fen*	Af2-F	GAATAYMTCGGMCGTMTKGA	443, 452/no	Stein (2005)
		Tf1-R	GCTTTWADKGAATSBCCGCC		
Bacillomycin	*bmyA*	bmyA_F	CTC ATT GCT GCC GCT CAA TC	853 bp/yes	Compaoré et al. (2013)
		bmyA-R	CCG AAT CTA CGA GGG GAA CG		
Bacilysin	*bacA/B*	bacA/B_F	TGC TCT GTT ATA GCG CGG AG	910 bp/yes	Compaoré et al. (2013)
	bac B	bacA/B_R	GTC ATC GTA TCC CAC CCG TC		

Table 2 In vitro inhibitory effect (%) of ZO4, RS11 and SF82 against *Verticillium dahliae, Neofusicoccum mediterraneum, Phytophthora* sp., *Botryosphaeria dothidea* and *Fusarium pseudograminearum*

Pathogens	Inhibitory effect of bacterial isolates (%)*		
	ZO4	RS11	SF82
Verticillium dahliae	72.82 ± 0.76 a,A	43.80 ± 0.20 b,A	47.87 ± 0.31 c,A
Neofusicoccum mediterraneum	70.17 ± 0.76 a,B	54.67 ± 0.71 b,B	46.87 ± 1.06 c,A
Phytophthora sp.	52.60 ± 0.76 a,C	42.50 ± 0.50 b,C	50.30 ± 0.6 c,B
Botryosphaeria dothidea	66.73 ± 0.76 a,D	62.13 ± 0.76 b,D	68.57 ± 0.67 c,C
Fusarium pseudograminearum	63.77 ± 0.76 a,E	66.60 ± 0.53 b,E	63.17 ± 0.76 c,D

*Values represent the average ± SD of three replicates. Values followed by different letters are significantly different according to Duncan's test at $p = 0.05$. The small letters indicate significant difference between bacterial isolates for the same pathogen. The capital letters indicate significant difference between pathogens for the same bacterial isolate. The data presented are from representative experiments that were repeated twice with similar results

number of plants with the severity of *i*, and N is the total number of plants. The $AUDPC_s$ was then calculated as the area under the curve of DI over time.

Evaluation of plant growth promotion of BCAs

The experiment was carried out on young pepper plants (2 months) using the dipping method. Roots were dipped in BCAs suspensions (10^8 CFU) for 30 min prior to planting. Control plants were dipped in sterile distilled water (Karlidag et al. 2007). Growth promoting effects were evaluated by measuring the length (cm) of stem and roots, and the weight (fresh and dry) of leaves, stem and roots (g).

All the experiments above were performed in three replicates.

Statistical analysis

Statistical analysis was performed using SPSS software (version 20). A completely randomized design was used for all experiments with 3 replications for each treatment. The data presented are from representative experiments that were repeated twice with similar results. Treatments were compared via ANOVA using the Duncan's test at 5% ($P = 0.05$) probability level.

Results

Biochemical and molecular identification of the BCA's

A collection of one hundred and sixty two isolates obtained from the rhizosphere of South Tunisian Oasis was conserved in the Microorganisms and active biomolecules lab. According to dual culture assay and well diffusion method, only thirty five isolates showed an antifungal activity against the 5 tested pathogens (data not shown). Among these, the three selected BCAs: ZO4, RS11 and SF82 were the most effective, particularly against *V. dahliae*, which growth has been inhibited at 72.82 ± 0.76%, 43.80 ± 0.20% and 47.87% ± 0.31%, respectively (Table 2). The three BCA isolates were first identified as *B. subtilis* by the Biolog system with a similarity index ranged between 0.514 and 0.646. Characteristics of biochemical reactions obtained from GENIII are described in Table 3. However, 16S rDNA analysis revealed 100% similarity of both RS11 (KX179571) and SF82 (KX179571) with *B. amyloliquefaciens* isolate (KX179573) and 100% similarity of ZO4 (KX179573) with *B. subtilis* isolate (MF581450).

Growth promoting traits and hydrolytic enzymes

Potential PGPR mechanisms of the selected BCAs were evaluated in vitro based on IAA production in chemically defined medium, phosphate solubilization in agar plate, and HCN, ammonia and hydrolytic enzymes (α amylase, protease, cellulose, mannanase) production. Results summarized in Table 4, revealed that the best activity was recorded for ZO4 isolate, which exhibited the highest amount of produced IAA (28.84 ± 0.27 μg ml^1) as well as a positive reaction for the other traits.

Pathogenicity test

One month after inoculating pepper plants with *V. dahliae* suspension, symptoms of fungal attack started to be gradually observed until becoming very clear by the end of the fifth week; a brownish color was observed on almost all leaves of affected branches; which became curly with weakened bases, leading to their easy fall to the simple touch. All inoculated plants died two months after inoculation whereas controls remained totally healthy (Fig. 1). Koch's postulate was then verified.

"In planta" antagonism against Verticillium disease

After treatment by BCA's cultures, the $AUDPC_s$ and the analysis of variance showed statistically significant differences in disease severity resulting especially in a total inhibition by ZO4 isolate (Fig. 2).

Table 3 Biochemical analysis of ZO4, RS11 and SF82 using the BIOLOG™ GEN III microplate

Properties	Results		
	ZO4	RS11	SF82
Negative control	–	–	–
Dextrine	+	+	+
D-Maltose	+	+	+
D-Trehalose	+	+	+
D-Cellobiose	+	+	+
Gentiobiose	+	+	+
Sucrose	+	+	+
D-Turanose	–	+	–
Stachyose	–	–	–
D-Raffinose	–	–	–
α-D-Lactose	+	+	+
D-Melibiose	–	–	–
β-Methyl-D-Glucoside	+	+	+
D-Salicin	+	+	+
N-Acetyl-D-Glucosamine	+	+	+
N-Acetyl-β-DMannosamine	–	–	–
N-Acetyl-D-Galactosamine	–	–	–
N-Acetyl Neuraminic Acid	–	–	–
α-D-Glucose	+	+	+
D-Mannose	+	+	+
D-Fructose	+	+	+
D-Galactose	–	–	–
3-Methyl-Glucose	–	–	–
D-Fucose	–	–	–
L-Fucose	–	–	–
L-Rhamnose	–	–	–
Inosine	+	–	–
Fusidic Acid	–	–	–
D-Sorbitol	+	+	+
D-Mannitol	+	+	+
D-Arabitrol	–	–	–
Myo-Inositol	+	+	+
Glycérol	+	+	+
D-Glucose-6-PO4	–	–	–
D-Fructose-6-PO4	+	+	+
D-Serine	–	–	–
Gelatine	–	–	–
Glycyl-L-Proline	–	–	–
L-Alanine	+	+	+
L-Arginine	+	+	+
L-Aspartic Acid	+	+	+
L-Glutamic Acid	+	–	–
L-Histidine	+	–	–
L-Pyroglutamic Acid	–	+	+
L-Serine	–	+	+
D-Galacturonic Acid	+	+	+
L-Galactonic Acid Lactone	–	–	–
D-Gluconic Acid	+	+	+
D-Glucuronic Acid	+	+	+
Mucic Acid	+	+	+
Quinic Acid	–	–	–
D-Saccharic Acid	–	+	+
p-HydroxyPhenylacetic Acid	–	–	–
Methyl-Pyruvate	+	–	–
D-Lactic Acid Methyl Ester	–	+	+
l-Lactic Acid	–	+	+
Citric Acid	–	+	+
α-Keto-Glutaric Acid	–	–	–
D-Malic Acid	+	+	+
l-Malic Acid	+	+	+
Bromo-Succinic Acid	+	+	+
Tween 40	–	–	–
γ-Amino-Butryric Acid	–	–	–
α-Hydroxy Butyric Acid	–	–	–

Table 3 (continued)

Properties	Results		
	ZO4	RS11	SF82
β-Hydroxy-d,l-Butyric Acid	+	–	+
α-Keto-Butyric Acid	–	–	–
Acetoacetic Acid	+	+	+
Propionic acid	–	–	–
Acetic acid	–	–	–
Positive control	+	+	+
pH 6	+	+	+
pH 5	+	+	+
1% NaCl	+	+	+
4% NaCl	+	+	+
8% NaCl	+	+	+
1% Sodium Lactate	+	+	+
Fusidic Acid	+	–	–
D-Sérine	–	+	+
Troleandomycine	+	+	–
Rifamycine SV	–	–	–
Minocycline	–	–	–
Lancomycine	–	–	–
Guanidine HCl	–	+	+
Niaproof 4	+	–	–
Vancomycin	–	–	–
Tetrazolium Violet	+	+	+
Tetrazolium Blue	–	–	–
Nalidixic Acid	–	–	–
Lithium Chloride	+	+	+
Potassium tellurite	+	+	+
Sodium butyrate	+	+	+
Sodium bromide	–	–	–
Gram	+	+	+
Oxydase	+	+	+
Catalase	+	+	+

(+): positive reaction; (−): negative reaction

Growth promoting effect in pepper plants

As presented in Table 5, all isolates significantly increased the length and the weight (fresh and dry) of leaves, stem and roots compared to non-inoculated controls. The analysis of variance showed significant differences between the BCAs ($p = 0.05$) as *B. subtilis* ZO4 has been proved to be the most efficient (Fig. 3).

Effect of enzymes, heat and pH on antimicrobial activity

Results showed that the incubation of CFS at different pH ranges reduced the antifungal activity of ZO4, RS11 and SF82 from 87% to 73%, 78% to 82%, and 67% to 72%; respectively at pH 4 and 10 (data not shown). In addition, they also revealed that the substance(s) produced by the BCAs

Table 4 Plant growth promoting traits of ZO4, SF82 and RS11 (In vitro *assay)*

BCAs		Hydrolytic enzyme production				Growth promoting traits		Phosphate solubilization	HCN production
	Urease	Protease	Cellulase	Mannanase	α- amylase	(IAA) μg/ml*	NH_3 production		
ZO4	+	+	+	+	+	28.84 ± 0.27	+	++	+
RS11	+	+	–	–	+	24.74 ± 0.37	+	+	+
SF82	+	+	+	–	+	27.08 ± 0.15	+	++	+

*Values represent the average ± SD of three replicates

(−): negative activity, (+): positive activity, (++): Diameter greater than 1.5 cm

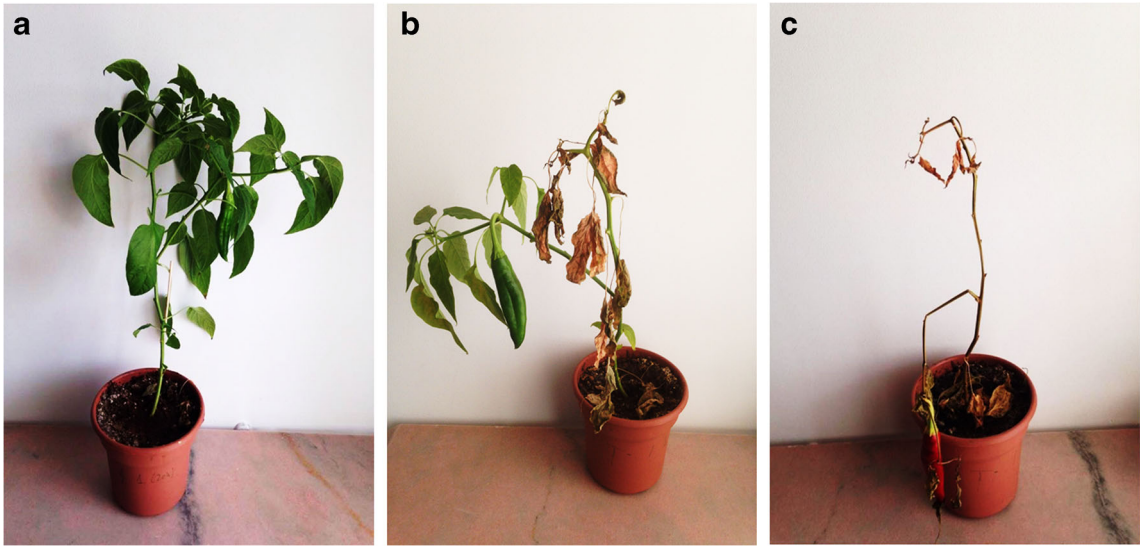

Fig. 1 Pathogenicity of *Verticillium dahliae* on 4 month years old pepper plants. a: negative control; b: symptoms observed 1 month after inoculation; c: symptoms observed after 2 months

showed sensitivity toward all the used proteolytic enzymes (Table 6). Furthermore, following 30 min exposure to wide range temperatures (50–120 °C), the activity of BCAs decreased then was completely lost at 120 °C.

PCR detection of lipopeptide biosynthesis genes

All the isolates ZO4, RS11 and SF82 were found to be negative for the genes involved in the biosynthesis of the lipopeptides surfactin (*srf*), fengycin (*fen*) and mycosubtilin (*myc/itu*). However, they contained genes involved in the biosynthesis of the lipopeptides iturins (*itu*A, *itu*D, *itu*C), bacilysin (*bac*A / *B-F, bac*A / *B-R*) and bacillomycin (*bmy*A) (Fig. 4).

Discussion

The best sources of antagonistic microorganisms are their natural environments, where they compete with naturally colonized microbiota that includes plant pathogens or spoilage microorganisms. This study was conducted to screen bacterial strains isolated from an oasis soil against verticillium wilt of pepper caused by *V. dahliae.* Of one hundred and sixty-two isolated bacteria, only three showed remarkable activity against the pathogen. All Three BCAs were identified by BIOLOG test as *B.subtilis*. Several studies have reported the use of BIOLOG micro plate assay for bacterial biocontrol agents, particularly in defining their specific carbon sources (Altinok et al. 2013; Ahmad et al. 2017). However, based on

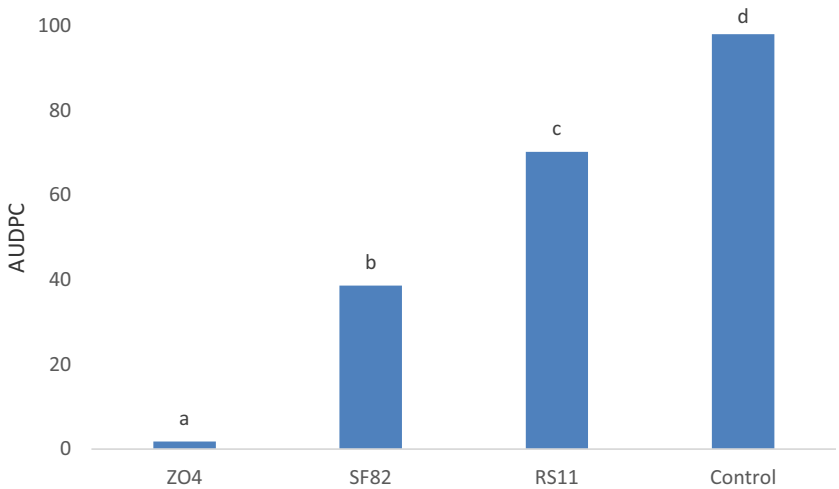

Fig. 2 Area under the disease progress curve on pepper plants (2 month years old) after inoculation with *Bacillus Subtilis* ZO4, *Bacillus amyloliquefaciens* SF82 and *Bacillus amyloliquefaciens* RS11. Means were compared by Duncan's test at $P = 0.05$

Table 5 Plant growth promoting effect of ZO4, SF82 and RS11 (*In planta asay)*

	Length(cm)*		Weight(g)*					
	Stem	Root	Stem		Leaf		Root	
			Fresh	Dry	Fresh	Dry	Fresh	Dry
Control	12.3 ± 0.58 a	4.73 ± 0.64 a	1.38 ± 0.06 a	0.17 ± 0.02 a	1.39 ± 0.10 a	0.15 ± 0.01 a	0.16 ± 0.03 a	0.06 ± 0.01 a
ZO4	22.9 ± 0.99 b	8.93 ± 0.21 b	2.94 ± 0.06 b	0.39 ± 0.04 b	3.84 ± 0.36 b	0.50 ± 0.04 b	0.62 ± 0.02 b	0.15 ± 0.01 b
RS11	15.3 ± 0.58 c	5.93 ± 0.31 c	1.35 ± 0.07 a	0.16 ± 0.01 a	1.81 ± 0.01 a	0.37 ± 0.19 ab	0.24 ± 0.01 c	0.09 ± 0.00 c
SF82	19.0 ± 0.76 d	7.03 ± 0.25 c	2.92 ± 0.09 b	0.38 ± 0.02 b	3.14 ± 0.17 c	0.40 ± 0.02 ab	0.55 ± 0.02 d	0.10 ± 0.00 c

*Values represent the average ± SD of three replicates. Values followed by different letters are significantly different according to Duncan's test at p = 0.05. The small letters indicate significant difference between bacterial isolates for the same column. The data presented are from representative experiments that were repeated twice with similar results

their 16S rRNA sequences, they were identified as *B. subtilis*: ZO4 and *B. amyloliquefaciens*: RS11 and SF82. These results are consistent with other studies that confirm a clear uncertainty provided by the GENIII software for genus and species level identifications (Wragga et al. 2014).

Antagonistic activity of the bacterial isolates was first evaluated in vitro, based on their ability to inhibit mycelia growth. According to the diffusion well assay against *Neofusicoccum mediterraneum*, *Phytophthora* sp., *Fusarium pseudograminearum*, *Botryosphaeria dothidea* and *Verticillium dahliae*, results revealed that bacterial culture filtrates were found to be highly active against all tested fungi (> 42%) with a significant difference between bacteria isolate for the same pathogen as well as between pathogens for the same bacterial isolate. *B. subtilis* ZO4, in particular, recorded the highest growth inhibition against *V. dahliae* (72.8%). These findings are consistent with several studies indicating that rhizosphere may be a common source for the selection of *Bacillus* species with important potentials that are useful for the biocontrol of both soil-borne and foliar pathogenic fungi (Govindasamy et al. 2010; Hinarejos et al. 2016). Moreover, on the basis of these results, the antibiotic activities exhibited by our BCAs appeared to be extracellular and easily recovered in their supernatant, which could be explained by the production of antifungal metabolites and lytic enzymes that are able to penetrate the cells of the pathogen and chemically inhibit its growth. Several authors have reported the large spectrum of antifungal activity of *Bacillus* spp. (Yang et al. 2014; Han et al. 2015a, b; Jahanshir et al. 2016) and have suggested that antibiosis could be the most common mode of antagonism observed among these species (Edwards and Seddon 2008). Other studies have also reported that *Bacillus* spp. protect plants through a number of mechanisms, particularly through the synthesis of different lipopeptides with inhibitory activity against phytopathogens (Falardeau et al. 2013; Cawoy et al. 2015; Torres et al. 2016)

The beneficial plant–microbe interactions in the rhizosphere are known to be important determinants of plant health and soil fertility (Pii et al. 2015). For this matter, our isolates were then tested for their different PGPR traits. Results

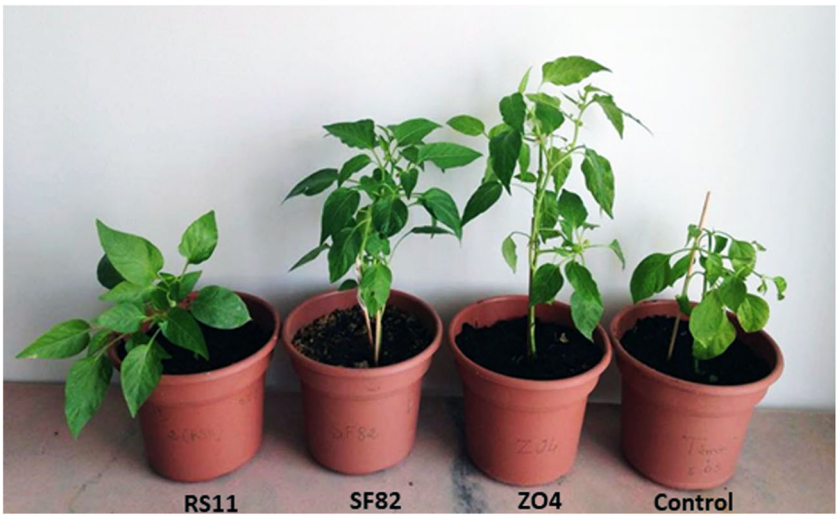

Fig. 3 Plant growth promoting effect of the BCAs on 2 month years old pepper plants

Table 6 Effect of enzymatic treatment on antifungal activity

Enzymatic treatment	Residual activity (%)*		
	ZO4	RS11	SF82
Proteinase K	25,33 ± 0,58 a	33,00 ± 1,00 b	50,00 ± 1,00 c
Lysozym	46,00 ± 1,00 a	46,00 ± 1,00 a	0
Trypsin	54,00 ± 1,00 a	86,00 ± 1,00 b	75,00 ± 1,00 c
α-Chymotrypsin	16,00 ± 1,00 a	73,00 ± 1,00 b	75,00 ± 1,00 c
Pepsin	41,00 ± 1,00 a	53,00 ± 1,00 b	63,33 ± 0,58 c
Peptidase	54,00 ± 1,00 a	28,00 ± 1,00b	37,33 ± 0,58 c

*Values represent the average ± SD of three replicates. Values followed by different letters are significantly different according to Duncan's test at p = 0.05. The small letters indicate significant difference between bacterial isolates for each treatment. The data presented are from representative experiments that were repeated twice with similar results

showed that all BCAs were IAA producers. Several studies reported the production of IAA by rhizobacteria isolated from corn, wheat and rice cultures (Cakmakci et al. 2007; Mehnaz et al. 2010); others have demonstrated that IAA enhances plant cell elongation and cell division which stimulates better the root growth (Dey et al. 2004; Gray and Smith 2005; Li et al. 2018). Moreover, results revealed that all isolates were able to solubilize phosphate and to produce HCN similarly to the findings of Dinesh et al. (2013). The production of HCN plays an important role in the biological control of plant pathogens. In fact, Blumer and Hass (2000) suggested that HCN could be proposed as a defense regulator against pathogenic diseases, such as wheat planting (Rana et al. 2011), since it acts as an indicator of the strain resistance. Furthermore, experiments showed that BCAs were able to produce ammonia, which has been reported in many studies (Singh et al. 2017; Vimal et al. 2018). Many researchers have also reported the involvement of such attributes in root and stem length (Beneduzi et al. 2012), which is consistent with our findings. In fact, the inoculation of pepper plants with BCAs revealed an important increase in their root and stem length compared to the control. A significant increase was also recorded for both fresh and dry leaf, stem and root weight. The Analysis of variance showed a significant difference between the bacterial isolates. Overall, the highest values were recorded by *B. subtilis* ZO4 isolate. These results are consistent with several studies that reported the importance of *Bacillus* spp. and especially *B. subtilis* as PGPR bacteria (Govindasamy et al. 2010; Yu et al. 2011; Xu et al. 2016).

Another mechanism interfering with fungal inhibition is the degradation of their cell wall by the action of lytic enzymes. Results revealed that BCAs degraded cellulose, mannanose, protease and amylase. Such findings are consistent with other studies which reported that these enzymes play an important role in fungal inhibition and in disease prevention through their capacity to degrade the fungal cell membrane, (Thabti et al. 2016).

Selected proteolytic enzymes, including Proteinase K, α–chymotrypsin and pepsin showed strong effect on the three

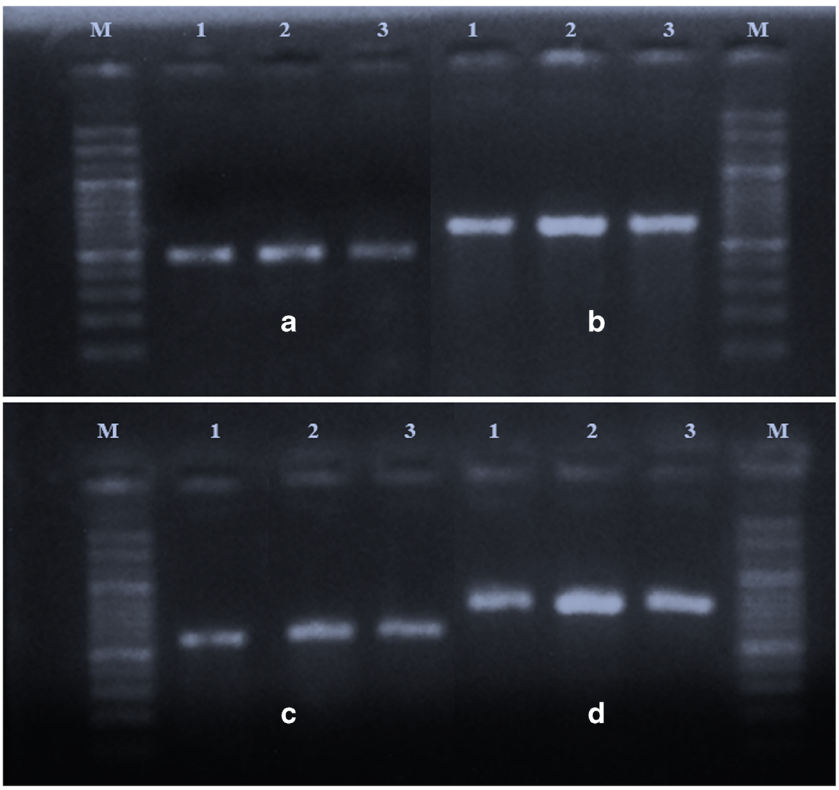

Fig. 4 Electrophoretic DNA banding patterns following the polymerase chain reaction of the lipopeptide biosynthesis genes. **M:** 100 bp; **(1):** SF82; **(2):** RS11; **(3):** ZO4; (**a**): *itu*D, (**b**): *itu*A, (**c**): *itu*C, (**d**):*bmy*A

CFS. This was highlighted by the significant loss of antifungal activity against *V. dahliae* compared to the control. For instance, antifungal activity of SF82 was completely lost after treatment with Lysozyme. Such results suggest that BCAs antifungal activities seem to be probably attributed to some protease sensitive compounds such as iturin group of antibiotics (Torres et al. 2016). Several studies have reported the proteinaceous characteristics of antimicrobial substances produced by *Bacillus* species (Compaoré et al. 2013; Ceresa et al. 2016; Caulier et al. 2018). On the other hand, inhibitory substances were found to be active in a wide pH range (4 to 10), and highly stable after heat treatment (resistance up to 100 °C). Numerous studies have reported the importance of such properties in biological control of soilborne pathogens, particularly under field conditions (Han et al. 2015a, b; Nawaz et al. 2018)

The detection of lipopetide synthesis genes has shown that our isolates are capable of producing iturin A, bacilysin and bacillomycine. These results are consistent with those of Compaoré et al. (2013). Bacillomycin, belong to the Iturin family, known for its antifungal properties, particularly against filamentous fungi which allow their application as bio-control agents (Liu et al. 2011; Zeriouh et al. 2011). Several studies have also shown the ability of these lipopeptides to inhibit fungi such as *V. dahliae* (Zhao et al. 2017).

It is well known that, unlike in vitro tests which only involve the pathogen and the antagonist under natural conditions, the role played by the host plant is crucial (Anith et al. 2003). Therefore, a "screening" system involving the infectious agent, the antagonist and the host, is necessary in order to have better information regarding the true inhibitory power of the selected BCAs. The *in planta* biological control experiment undertaken in this study, revealed variable antagonistic activities of the three BCAs. In fact, wilt severity in the form of AUDPC was significantly affected by treatments. Both *B. amyloliquefaciens* SF82 and RS11 have significantly reduced the appearance of wilt symptoms. However, especially plants treated with *B. subtilis* ZO4 strain sustained significantly the lowest AUDPC value (AUDPC = 1.8) compared to the control (AUDPC = 98). Such disease suppression requires further research, mainly focused on ZO4 mechanism and plant roots interaction. Previous studies have proposed that root colonization by biological agents plays an important role in the suppression of soil pathogens (Compant et al. 2005; Cao et al. 2011). In fact, as reported by Haggag and Timmusk (2008), the colonization of roots by BCAs before pathogen establishment, enhance biological control by preventing pathogen penetration; moreover, fast colonization of roots could be an important factor for the establishment and the introduction of BCAs in the rhizosphere and thus for biocontrol efficacy (Gamalero et al. 2003). Several studies have confirmed the antifungal activity of *Bacillus* spp. and their role in increasing plant yield. For instance, Tjamos et al. (2004) reported the isolation of a *B. amyloliquefaciens* strain that significantly reduced verticillium disease of apple trees and increased the yields up to 25%.

In conclusion, this study has shown the potential of ZO4, RS11 and SF82 rhizobacterial strains as important biological control and PGPR agents capable of reducing or eliminating symptoms of verticillium wilt in pepper plants. Especially, *B. subtilis* ZO4 isolate seems to be a promising candidate. However, its application under field conditions needs more investigations regarding its concentration, formulation and delivery method.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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