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Endophytic *Bacillus* spp. from Wild Solanaceae and Their Antifungal Potential against *Fusarium oxysporum* f. sp. *lycopersici* Elucidated Using Whole Cells, Filtrate Cultures and Organic Extracts

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Abstract

Six isolates of culturable bacteria, isolated from stems of wild Solanaceae species (*Datura metel*, *Solanum nigrum*, *S. elaeagnifolium*, and *Nicotiana glauca*), were assessed for their antifungal activity against *F. oxysporum* f. sp. *lycopersici* (FOL), the causal agent of the tomato Fusarium wilt. Blast analysis of 16S rDNA sequencing genes homology showed that the isolates belonged to the genus *Bacillus* (*Bacillus cereus* str. S42, *B. tequilensis* str. SV39, *B. subtilis* str. SV41, *B. methylotrophicus* str. SV44, *B. amyloliquefaciens* subsp. *plantarum* str. SV65, and *B. mojavensis* str. SV104). The mycelium growth of FOL was significantly reduced by 36 to 46% by diffusible metabolites and by 18 to 21% by volatile compounds. Cell-free cultures were found to be mostly active when issued from 4 days-old cultures where FOL growth inhibition significantly varied from 31.1 to 59.5%. Active metabolites present in the cell-free cultures were extracted with n-butanol and chloroform. Both organic extracts exhibited antifungal potential towards FOL higher than that induced by the two commercial products i.e. Bavistin® (50%, chemical fungicide) and Bactospeine® (16000UI/mg, biopesticide). This study clearly indicates that endophytic *Bacillus* spp. from wild Solanaceae species can be used as natural sources of bioactive metabolites towards FOL. Wild Solanaceae are frequent in Tunisia and were not explored as potent sources of candidate antagonistic bacteria. In view of the endogenous progress of the pathogen via the vascular tissues, the use of endophytic bacteria can suppress tomato Fusarium wilt disease.

Keywords: Antifungal activity; *Bacillus* spp.; Cell-free cultures; *Fusarium oxysporum* f. sp. *lycopersici*; Metabolites; Organic extracts

Introduction

Fusarium oxysporum f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hans is one of the most important pathogens infecting tomato worldwide [1,2]. *Fusarium oxysporum* f. sp. *lycopersici* (FOL) is a widespread pathogen and wilt severity depends on regional cultural practices [3]. This pathogen is responsible for important crop losses both in open field and protected crops [1,4,5].

Use of resistant cultivars, crop rotation and solarization have been widely used for the control of soilborne fungal diseases but they did not give satisfactory results [6]. Moreover, due to the development of resistance mutations and due to the emergence of new physiological races, many of synthetic chemicals fungicides and resistant cultivars are gradually becoming ineffective in controlling the disease [7].

The control of Fusarium wilt of tomato is so difficult due to the endogenous progress of the pathogen via the vascular tissues [8] and to the limited range of effective fungicides [9]. Besides, the long survival of chlamydospores in the soil, for extended periods without a host, limited the suppressive effects of crop rotation [1,8]. Added to these constraints, the resistance of tomato cultivars to races 1 and 2 of FOL, which has long been adopted as an effective and safe alternative to human and the environment, was overcome by the emergence of the race 3 of the pathogen in several countries [5,10]. Research studies have been more focused on sources of genetic resistance to this emergent race and on alternative practices for achieving an effective control of this pathogen [10].

Therefore, there is an increasing interest in the development of control alternatives.

Biological control was considered as an environmentally safe alternative. Given the endogenous progress of the pathogen within plant tissues, the use of endophytic microorganisms could better limit the disease [11-13].

Endophytic bacteria, used as whole cells [14,15], cell-free culture filtrates and/or organic extracts [16,17] gave satisfactory results in controlling some plant pathogenic fungi. These biocontrol agents (BCAs) have been isolated from a variety of plants because they ubiquitously inhabit mostly wild species such as *Prosopis strombulifera* [18], *Huperzia serrata* [19], *Suaeda maritima*, *Carex scabrifolia*, and *Elymus mollis* [20]. Recently, a wild Solanaceae, *Nicotiana glauca*, was

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used as natural source of beneficial leaf-associated bacteria, especially *Bacillus* spp. [21].

Previous studies used endophytic bacteria as BCAs against plant pathogenic fungi such as *F. oxysporum* f. sp. *cubense*, *Colletotrichum gloeosporioides*, *Alternaria alternata*, *Botrytis cinerea*, *B. fabae*, *Pythium ultimum*, *Rhizoctonia solani*, *Verticillium dahliae*, *Sclerotium rolfsii*, *Sclerotinia sclerotiorum* and *Penicillium digitatum* [15,22,23].

Therefore, searching for new bioactive metabolites from endophytic bacteria is a new and safe way of controlling plant diseases [16,24]. These metabolites from *Bacillus* species include lipopeptide antibiotics [12,25], cell-degrading enzymes [14,26] and other substances belonging to esters, ketones, alcohols, aldhehydes and phthalic acids families [27].

In this study, endophytic bacteria isolated from healthy stems of wild Solanaceae plants (*N. glauca*, *D. metel*, *S. elaeagnifolium* and *S. nigrum*) were identified and tested *in vitro* for their antifungal activity against FOL. Their cell-free culture filtrates and organic extracts were also evaluated for their suppressive effects towards FOL.

Materials and Methods

Pathogen isolation and culture

The isolate of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) used in this study was originally isolated from tomato plants exhibiting typical symptoms of Fusarium wilt. Stem sections (3-5 cm in length) showing vascular discoloration were rinsed thoroughly with tap water. After surface-disinfesting in sodium hypochlorite solution (5%) for 2 min, the stem pieces (1 cm in length) were rinsed three times with sterile-distilled water (SDW) and dried on sterile filter paper. They were plated onto Potato Dextrose Agar (PDA) medium amended with streptomycin sulfate (300 mg/L) (w/v). Fungal cultures were incubated for one week at 25°C. The fungal isolate was cleaned up by subculturing successively and selected by single-spore isolation. The isolate of FOL selected was re-isolated from artificially inoculated tomato cv. Rio Grande plants fulfilling Koch's postulates and incubated at 25°C for 7 days before use.

Plant sampling and endophytic bacteria isolation and culture

Healthy wild Solanaceae species (*N. glauca*, *D. metel*, *S. nigrum* and *S. elaeagnifolium*) growing spontaneously nearby tomato fields with a history of severe soilborne diseases, were used for isolation of endophytic bacteria. Stems were sampled, at the fruiting stage, on April and November 2013 from different ecological sites of the Tunisian Centre-East (Chott-Mariem, Bekalta, M'saken). Stems were washed with tap water and processed for endophytic bacteria isolation as follows.

Five stem Samples (5 cm in length) were individually disinfected by soaking into 70% ethanol for 1 min, immersion in 1% sodium hypochlorite solution for 10 min then in 70% ethanol for 30 s. They were rinsed three times in SDW and air-dried on sterile filter papers. After check for the efficiency of the surface sterilization procedure according to McInroy and Kloepper [28], two methods of endophytic bacteria isolation were used. The first one consisted of transferring aseptically twenty pieces (1 cm in length) of sterile stems onto Nutrient Agar (NA) medium. This method was used to isolate endophytic bacteria from surface-sterilized stems. In the second method, three stem pieces were pierced with a sterile-nipper and the liquid exuding from the internal tissues was streaked on NA. This method was used to isolate endophytic bacteria from internal tissues of stems (Table 1). Before be used in the different bioassays, stored cultures in NA supplemented with 40% glycerol at -20°C were subcultured on NA and incubated at 25°C for 48 h.

Characterization and hypersensivity test of endophytic bacterial isolates

Colonies of bacterial isolates were observed macroscopically and characterized based on their size, shape, margin, elevation, texture, opacity, consistency and pigmentation on NA. Morphology, mobility and Gram's staining of culturable isolates were performed using light microscopy [29]. Isolates were also characterized using conventional biochemical tests according to Schaad et al. [30] protocols.

To check the non-pathogenicity of the isolates tested, 10 µl of bacterial suspension (~10^8 cells/mL) was inoculated to tobacco leaves using a syringe. Uninoculated control leaves were treated with SDW only (negative control). Tobacco plants (inoculated and uninoculated) were incubated at room temperature for 24 h. Isolates inciting the development of chlorotic and/or necrotic zone on leaf areas were considered as pathogenic and thus, excluded from their eventual screening as BCAs [31].

Identification of endophytic bacterial isolates by 16S rDNA sequencing gene

Molecular identification of the bacterial isolates was performed after extraction of the genomic DNA using the method described by van Soolingen et al. [32] for Gram+ bacteria. The 16S rDNA was amplified with the universal eubacterial primers 27f (5'-AGAGTTTGATC(A/C)TGGCTCAG-3') and 1492r (5'-TACGG(C/T)TACCTTGTTACGACTT-3') [4]. Amplifications were carried out in Thermal Cycler (CS Cleaver, Scientific Ltd., TC 32/80). The cycling conditions were as follows: one denaturing cycle at 94°C for 4 min, followed by 40 denaturing cycles at 94°C for 30 s, annealing at 45°C for 30 s, and polymerization at 72°C for 45 s. The amplification was terminated with an extension cycle at 72°C for 7 min. The homology of the 16S rDNA sequence of a given isolate was performed using BLAST program from Genbank database (http: www.ncbi.nlm.gov/BLAST/). The six culturable endophytic bacteria (namely S42, SV39, SV41, SV44, SV65, and SV104) sequences were submitted to GenBank and were assigned the following accession numbers KP993206 KR818070, KR818071, KR818072, KR818073 and KR818089, respectively.

In vitro antifungal activity test of endophytic *Bacillus* spp. isolates

Dual culture method: The antagonistic potential of *Bacillus* spp. isolates against FOL was evaluated using the dual culture method on PDA. This method consists to streak bacterial isolates across the center of the Petri plate (9 cm in diameter) and perpendicularly to the

Isolate	Plant	Source of bacterial isolation	Locality	GPS locality
S42	*Nicotiana glauca*	Stems	Bekalta	N35°37'14.327"; E10°59'41.393"
SV39	*Datura metel*	Internal tissues of stems	Chott-Mariem	N35°56'20.451"; E10°33'32.028"
SV41	*D. metel*	Internal tissues of stems	Chott-Mariem	N35°56'20.451"; E10°33'32.028"
SV44	*D. metel*	Internal tissues of stems	Chott-Mariem	N35°56'20.451"; E10°33'32.028"
SV65	*Solanum nigrum*	Internal tissues of stems	Chott-Mariem	N35°56'20.451"; E10°33'32.028"
SV104	*S. elaeagnifolium*	Internal tissues of stems	M'saken	N35°43'32.073"; E10°34'48.90"

Table 1: Endophytic bacterial isolates from wild Solanaceae plants and their isolation sources.

first streak. Four agar-plugs (6 mm in diameter), removed from the growing edge of a 7 day-old culture of FOL, were placed at each side of the tested bacterial isolate [33]. The control plates were streaked with SDW only. Each individual treatment was repeated four times. After 4 days of incubation at 25°C, the colony diameter of the pathogen was measured. The mycelial growth inhibition rate of the pathogen (IR) was calculated using the formula of Dennis and Webster [34] as follows: IR %=[(C2-C1) / C2] × 100 where C2: Mean diameter (two perpendicular measurements) of the control colony and C1: Mean pathogen colony diameter in the presence of the antagonist.

Sealed plate method: This method of confrontation permits the detection of volatile metabolites produced by the bacterial isolate tested against FOL. *Bacillus* spp. were streaked onto NA in the bottom of Petri dish plate. An agar plug (6 mm in diameter), containing mycelium taken from 7-day-old pathogen culture, was placed in the center of a second Petri dish plate containing PDA amended with streptomycin sulfate (300 mg/L) (w/v). The plate containing the pathogen mycelial plug was inverted over the bacterial plate and these two dishes were sealed with Parafilm in order to avoid evaporation of volatile compounds. In the control culture, the Petri dish below contained only NA without streaking bacteria [35]. The plates were incubated at 25°C for 7 days. Each individual treatment was repeated thrice. The mycelial growth inhibition rate of the pathogen was calculated as previously described.

In vitro antifungal activity test of cell-free culture filtrates of endophytic *Bacillus* spp. isolates

To determine the relationship between growth and production of antifungal metabolites, each *Bacillus* spp. isolate was cultured in Luria-Bertani broth (LB) medium at 28 ± 2°C for 1, 2, 3, 4 and 7 days and under continuous shaking at 150 rpm. Culture samples of 2 ml each were taken at various time points and examined for their antifungal activity. Liquid cultures obtained were centrifuged for 10 min at 10,000 rpm. The centrifugation was repeated three times. Supernatant fluids were sterilized by filtration through a 0.22 µm pore size filter. The control was the LB filtrate. The filtrates were added at the concentration of 10% (v/v) aseptically to Petri dishes containing PDA amended with streptomycin sulfate (300 mg/L) (w/v). After solidification of the mixture, three agar plugs of the pathogen (6 mm in diameter) were plated equidistantly in each Petri plate. Fungal cultures were incubated at 25°C for 4 days [36]. The colony diameter of the pathogen (treated and untreated control) was measured and the mycelial growth inhibition rate of the pathogen was calculated as described above.

In vitro antifungal activity test of organic extracts from endophytic *Bacillus* spp. isolates

To extract the antifungal metabolites produced by *Bacillus* spp., two types of extraction were carried out: one with chloroform [37] and a second with n-butanol [38]. Sixty milliliters (60 mL) of cell-free culture of each isolate, prepared as described above, were placed in a separating funnel. Then, 60 mL of the solvent (chloroform or n-butanol) were added carefully. The funnel was reversed several times by degassing from time to time. The mixture was allowed to settle for few minutes with the cap open. The organic phase (the lower phase for extraction with chloroform and the upper one with n-butanol) were collected. The aqueous phase was replaced in the funnel and the extraction was repeated two other times. The solvent was evaporated in a rotary evaporator at 35°C for chloroform and 75°C for n-butanol with a slight rotation at 150 rpm.

The obtained organic extracts were assessed for their biological activity against FOL. Each extract was suspended in ethanol (1:1) (mg/mL) (w/v) and added to Petri dishes containing 10 ml of PDA amended with streptomycin sulfate (300 mg/L) (w/v) at two concentrations 2.5 and 5% (v/v). The control cultures were treated with ethanol only used at 2.5 and 5% (v/v). Antifungal activity of the metabolites produced by each isolate was compared to two commercial products i.e. Bavistin® (50%, chemical fungicide) and Bactospeine® (16000 UI/mg, biopesticide). Ethanol was used as negative control. After solidification of the mixture, a plug (6 mm in diameter) of FOL, obtained from PDA plate grown at 25°C for 7 days, was placed at the center of each plate. Fungal cultures were incubated for 7 days at 25°C. The colony diameter of the treated and control pathogen were measured and the mycelial growth inhibition rate of the pathogen was calculated as described above.

Statistical analysis

Data were subjected to a one-way analysis of variance using Statistical Package for the Social Sciences (SPSS) software for Windows version 16.0. For all the *in vitro* bioessays, each treatment was repeated three or four times. Data of the antifungal activity of whole cells and cell-free cultures were analyzed according to a completely randomized design. The *in vitro* essay of organic extracts was analyzed according to a completely randomized factorial model with two factors (treatments and concentrations). Means were separated using Student-Newman-Keuls test at $P \leq 0.05$.

Results

Characterization and hypersensivity test of endophytic bacterial isolates

All bacterial isolates were found to be Gram positive strains. The colony morphology of the six isolates showed an irregular form, rough surface and cream color on NA. These isolates were opaque. The isolate S42 showed flat elevation. SV39, SV41, SV44, SV65 and SV104 showed a humped elevation. A macroscopic variability was noted between the six isolates in terms of margin which was undulate (S42 and SV41), curly (SV39 and SV44), lobed (SV65) or irregular (SV104). Microscopically, the six isolates were rod-shaped and motile bacteria. They were able to produce catalase and indole by tryptophanase. SV39, SV41, SV44, SV65 and SV104 used mannitol as a carbon source. S42, SV41, SV44 and SV104 used also the simmons citrate as a carbon source. Except SV39, all isolates were able to synthesize the nitrate reductase. Only the S42 was able to produce the lecithinase. The six isolates were oxidative strains. They cannot ferment glucose through the mixed acid (MR) but by using the glycol butylene path (PV+). They cannot produce hydrogen sulfide, lysine decarboxylase and pyocyanin on King A medium. Only SV39 can produce urease. SV41 and SV44 can produce the tryptophane desaminase (Table 2).

The hypersensivity test onto tobacco plants revealed, after 24 h of incubation, that all the inoculated leaves remained healthy. No hypersensitive reaction (HR) (chlorotic or necrotic zone) was detected on the leaf areas as compared to the inoculated control leaves. Thus, all the isolates tested were non pathogenic and were selected for further molecular identification and screening of their antifungal activity against FOL.

Molecular identification of the bacterial isolates

Blast analysis of sequenced 16S rDNA gene homology revealed that the six endophytic isolates tested belonged to the genus of *Bacillus* with 100% of similarity to *B. cereus* with strain S42 (KP993206) and

Biochemical tests	Bacterial isolates					
	S42	SV39	SV41	SV44	SV65	SV104
Catalase	+	+	+	+	+	+
Red of Methyl	-	-	-	-	-	-
Vosges-Proskauer	+	+	+	+	+	+
Mannitol	-	+	+	+	+	+
Lecithinase	+	-	-	-	-	-
Indole	+	+	+	+	+	+
Simmons Citrate	+	-	+	+	-	+
Urease	-	+	-	-	-	-
Tryptophane desaminase	-	-	+	+	-	-
Nitrate reducatase	+	-	+	+	+	+
Glucose	+	+	+	+	+	-
Lactose	-	-	-	-	-	+
Gaz	+	+	+	+	+	-
Hydrogen sulfide	-	-	-	-	-	-
King A	-	-	-	-	-	-

Table 2: Biochemical characterization of the six bacterial isolates from wild Solanaceae stems. +: Positive test, -: Negative test. S42: Bacterial isolate from *Nicotiana glauca* stems; SV39, SV41 and SV44: Bacterial isolates from internal tissues of stems of *Datura metel*; SV65: Bacterial isolate from internal tissues of stems of *Solanum nigrum*; SV104: Bacterial isolate from internal tissues of stems of *S.elaeagnifolium*.

Isolate	Accession number	Most related species	Sequence homology (%)
S42	KP993206	265XY1, *Bacillus cereus*	100
SV39	KR818070	BD18-B03, *Bacillus tequilensis*	99
SV41	KR818071	264ZY7, *Bacillus subtilis*	99
SV44	KR818072	LK6, *Bacillus methylotrophicus*	99
SV65	KR818073	Hs8-12, *Bacillus amyloliquefaciens* subsp. *plantarum*	99
SV104	KR818089	ifo 15718, *Bacillus mojavensis*	99

Table 3: Identification of endophytic bacteria isolates by 16S rDNA sequencing genes.

99% of similarity to *B. tequilensis*, *B. subtilis*, *B. methylotrophicus*, *B. amyloliquefaciens* subsp. *plantarum* and *B. mojavensis* with isolates SV39 (KR818070), SV41 (KR818071), SV44 (KR818072), SV65 (KR818073) and SV104 (KR818089), respectively (Table 3).

Assessment of the *in vitro* antifungal activity of *Bacillus* spp. using whole cell suspension

Tested using the dual culture method in PDA, all *Bacillus* spp. isolates showed a significant inhibitory effect (at $P \leq 0.05$) against FOL, after 4 days of incubation at 25°C, compared to the untreated control. The reduction of FOL mycelium growth varied from 36.7 to 46.5%. The isolate SV65 of *B. amyloliquefaciens* subsp. *plantarum* inhibited FOL growth by 46.5% followed by 44.4, 44.1, 42.4, 38.7, and 36.7% recorded using the isolates SV41 of *B. subtilis*, S42 of *B. cereus*, SV44 of *B. methylotrophicus* (Figure 1a), SV104 of *B. mojavensis*, and SV39 of *B. tequilensis*, respectively (Table 4). This antifungal potential against of FOL may be attributed to diffusible metabolites produced by *Bacillus* spp.

Results shown in Table 4 revealed also that *Bacillus* spp. tested, using the sealed plate method, also exhibited a significant inhibitory effect (at $P \leq 0.05$) against FOL. Pathogen growth reduction varied significantly from 18.2 to 21.6% as compared to the untreated control. The antifungal effect induced by SV44 of *B. methylotrophicus* (Figure 1b) was expressed by 21.6% decrease in pathogen growth, as compared to the untreated control, followed by SV65 of *B. amyloliquefaciens* subsp. *plantarum* (20.3%), SV104 of *B. mojavensis* (20.8%), S42 of *B. cereus* (19.2%), SV39 of *B. tequilensis* and SV41 of *B. subtilis* (18.2%). This revealed the ability of endophytic *Bacillus* spp. to inhibit pathogen at distance through antifungal volatile compounds.

Assessment of the *in vitro* antifungal activity of *Bacillus* spp. using cell-free cultures

Analysis of variance revealed a significant (at $P \leq 0.05$) variation in the diameter of FOL colonies treated with the cell-free culture of *Bacillus* spp. tested at 10% (v/v) issued from 2-, 3-, 4-, and 7- day-old culture in LB medium at 28 ± 2°C. However, all cell-free culture

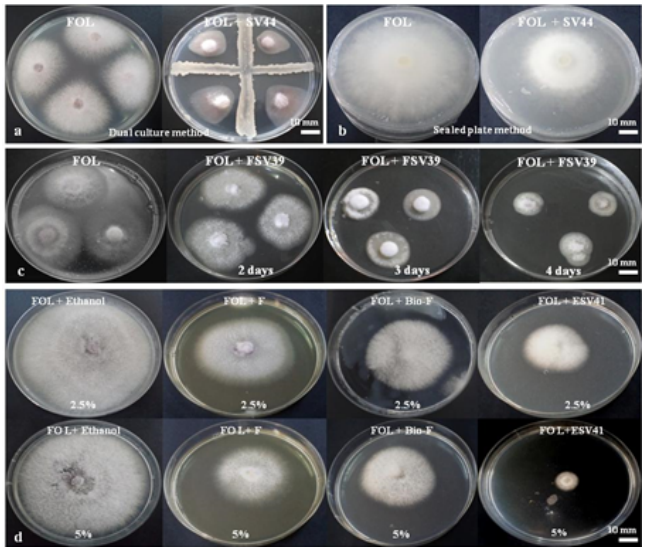

Figure 1: Antifungal activity of endophytic *Bacillus* spp. against *Fusarium oxysporum* f. sp. *lycopersici* attributed to diffusible (a) and volatile (b) metabolites, cell-free filtrates from 2 to 4-day-old bacterial cultures (c) and organic extracts tested at two concentrations (d) as compared to controls. SV44: Whole cell suspension of *Bacillus methylotrophicus* str. SV44; FSV39: Cell-free culture from *Bacillus tequilensis* str. SV39; Ethanol: Negative control; F: Positive control (Bavistin®, chemical fungicide); Bio-F: Positive control (Bactospeine®, Bio-pesticide); ESV41: Chloroform extract from *B. subtilis* str. SV41.

Bacterial treatments	Colony diameter (cm) and growth inhibition of FOL (%)	
	Diffusible metabolites	Volatile metabolites
Untreated control	3.71 a (0)	6.42 a (0)
Bacillus cereus str. S42 (KP993206)	2.07 b (44.1)	5.18 b (19.2)
Bacillus tequilensis str. SV39 (KR818070	2.35 b (36.7)	5.25 b (18.2)
Bacillus subtilis str. SV41 (KR818071)	2.06 b (44.4)	5.25 b (18.2)
Bacillus methylotrophicus str. SV44 (KR818072)	2.14 b (42.4)	5.03 b (21.6)
Bacillus amyloliquefaciens subsp. *plantarum* str. SV65 (KR818073)	1.99 b (46.5)	5.12 b (20.3)
Bacillus mojavensis str. SV104 (KR818089)	2.27 b (38.3)	5.08 b (20.8)

Table 4: Mycelial growth inhibition of *Fusarium oxysporum* f. sp. *lycopersici* by diffusible and/or volatile metabolites produced by endophytic *Bacillus* spp. isolates, as compared to the controls, noted after 4 and 7 days of incubation at 25°C, respectively. For each column, values followed by the same letter are not significantly different according to Student Newman Keuls test at $P \leq 0.05$. Numbers in parenthesis indicate the percentage (in %) of the mycelial growth inhibition of *Fusarium oxysporum* f. sp. *lycopersici* as compared to the untreated control.

Treatment tested	Duration of bacterial culture incubation (days)				
	1	2	3	4	7
Control	3.42 a	4.03 a	3.62 a	3.7 a	3.87 a
FS42	3.18 a (2)	3.58 a (11.2)	2.8 b (22.6)	2.55 b (31.1)	3.28 ab (15.2)
FSV39	3.35 a (2)	3.52 a (12.6)	2.22 b (38.7)	1.93 c (47.8)	2.38 b (38.5)
FSV41	3.13 a (8.5)	3.38 a (16.1)	2.37 b (34.5)	2.03 c (45.1)	2.48 b (35.9)
FSV44	3.18 a (7)	3.47 a (13.9)	2.53 b (30.1)	1.88 c (49.2)	3.03 b (21.7)
FSV65	3.18 a (7)	2.5 b (37.9)	1.97 b (45.6)	1.50 c (59.5)	2.33 b (39.8)
FSV104	3.08 a (9.9)	3.48 a (13.6)	2.02 b (44.2)	2.02 c (45.4)	2.47 b (36.2)

Table 5: Effect of cell-free cultures from endophytic *Bacillus* spp., prepared at different durations of incubation, against *Fusarium oxysporum* f. sp. *lycopersici* mycelial growth noted after 4 days of incubation at 25°C as compared to the untreated controls. FS42, FSV39, FSV41, FSV44, FSV65 and FSV104: Cell-free culture filtrates of isolates S42 of *B. cereus*, SV39 of *B. tequilensis*, SV41 of *B. subtilis*, SV44 of *B. methylotrophicus*, SV65 of *B. amyloliquefaciens* subsp. *plantarum* and SV104 of *B. mojavensis*, respectively. Control: Luria-Bertani broth medium filtrate. For each incubation duration, values followed by the same letter are not significantly different according to Student Newman Keuls test at $P \leq 0.05$. Numbers in parenthesis indicate the percentage (in %) of the mycelial growth inhibition of *Fusarium oxysporum* f. sp. *lycopersici* as compared to the untreated control.

filtrates issued from 1 day-old cultures did not decrease significantly (2 to 9.9%) pathogen growth. Cell-free cultures were found to be mostly effective when extracted from 4 days-old cultures where growth inhibition achieved varied from 31.1 to 59.5% as compared to 22.6-45.6%, 15.2-39.8%, and 11.2-37.9% recorded at 3, 7, and 2 days of incubation, respectively. For example, the cell-free culture of SV39 of *B. tequilensis*, issued from 4 days-old cultures, inhibited the pathogen growth by 47.8% whereas 38.7, 38.5, 12.6, and 2% were recorded using filtrates extracted after 3, 7, 2 and 1 day (s) of incubation, respectively (Table 5 and Figure 1c). The highest reduction (37.9, 45.6, 59.5, and 39.8%) of FOL mycelial growth was recorded using the filtrate of SV65 of *B. amyloliquefaciens* subsp. *plantarum* from 2-, 3-, 4-, and 7-days-old of cultures respectively, as compared to the controls (Table 5). Thus, the optimum incubation duration of *Bacillus* spp. cultures for the production of the most effective antifungal metabolites against FOL was found to be of about 3 to 4 days.

Assessment of the *in vitro* antifungal activity of organic extracts from endophytic *Bacillus* spp.

ANOVA analysis revealed a significant (at $P \leq 0.05$) variation in the average diameter of FOL colonies depending on the organic extracts (chloroform and n-butanol extracts) tested and the concentrations used, and the existence of a significant interaction between both factors. Results shown in Figure 2 indicated that all organic extracts, used 1 mg/mL (w/v), had inhibited FOL growth by 37 to 90% as compared to the ethanol controls. The decrease in FOL growth was higher with these organic extracts as compared to Bavistin® (31.3-39.5%) and Bactospeine® (40.9-43.2%) whatever the concentration used (Figures 1d and 2).

All organic extracts of *Bacillus* spp. were found to be more active at the concentration of 5% than at 2.5% (v/v), except the chloroform extract of S42 of *B. cereus*. In fact, all the organic extracts from the antagonistic agents used at 5% (v/v) had significantly reduced, by 57.4 to 90%, FOL growth compared to 37 to 72.4% recorded using 2.5% (v/v) concentration. Chloroform extracts of isolates SV65 of *B. amyloliquefaciens* subsp. *plantarum*, SV41 of *B. subtilis* (Figure 1d), and SV44 of *B. methylotrophicus* decreased pathogen growth by 73.02, 72.79, and 57.44%, respectively, when applied at 5% (v/v), compared to 43.4, 54.8 and 49.9% recorded at 2.5% (v/v) (Figure 2). In addition, n-butanol extracts of isolates SV104 of *B. mojavensis*, SV44 of *B. methylotrophicus*, and SV41 of *B. subtilis*, applied at 5%, inhibited the pathogen growth by 88.4, 88.6 and 83.2% compared to 37, 47.6 and 48.7% noted with the concentration 2.5% (Figure 2).

The highest inhibition (90%) was achieved using the n-butanol extract from SV65 of *B. amyloliquefaciens* subsp. *plantarum* followed by 88.6, 88.3 and 83.2% obtained with n-butanol extracts from SV44 of *B. methylotrophicus*, SV104 of *B. mojavensis*, and SV41 of *B. subtilis*, respectively used at 5% (v/v) as compared to the ethanol control (Figure 2). Tested at 2.5% (v/v), chloroform extracts from SV39 of *B. tequilensis* and S42 of *B. cereus* exhibited the highest antifungal potential towards FOL (72.4 and 71.7 %, respectively) (Figure 2).

Discussion

Bacillus spp. are known for their diverse range of secondary metabolites including antibiotics, lytic enzymes and volatile organic compounds with antifungal, antibacterial, nematicidal, insecticidal and immunosuppressant activities [39-42]. While a wide range of biologically active compounds have been isolated from endophytic

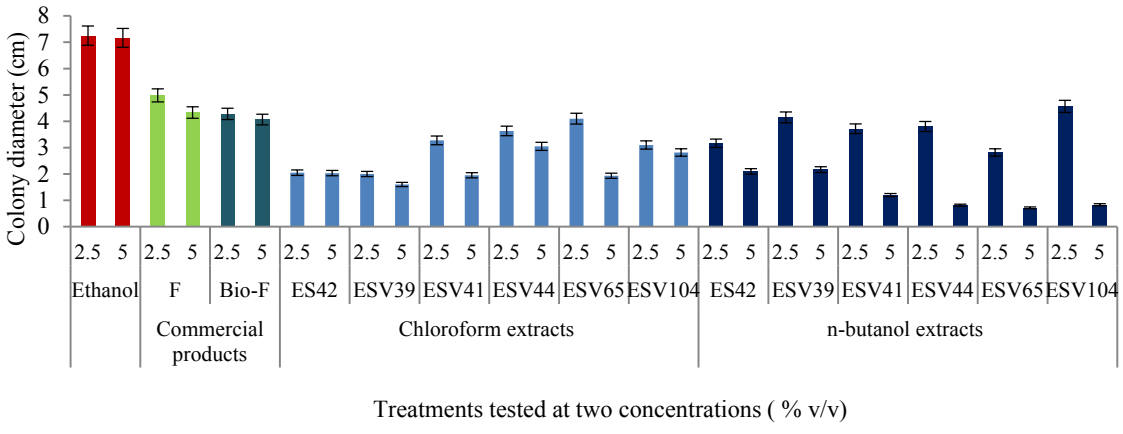

Figure 2: Effect of chloroform and n-butanol extracts from endophytic *Bacillus* spp. tested at two concentrations against *Fusarium oxysporum* f. sp. *lycopersici* noted after 7 days of incubation at 25°C as compared to the control and to the commercial products used. ES42, ESV39, ESV41, ESV44, ESV65 and ESV104: Organic extracts from isolates S42 of *B. cereus*, SV39 of *B. tequilensis*, SV41 of *B. subtilis*, SV44 of *B. methylotrophicus*, SV65 of *B. amyloliquefaciens* subsp. *plantarum* and SV104 of *B. mojavensis*, respectively; Control: Ethanol control. F: Bavistin® (Chemical fungicide); Bio-F: Bactospeine® (Bio-pesticide). LSD (Treatments tested × Concentrations used): 0.44 cm at $P \leq 0.05$.

organisms, they still remain a relatively untapped source of novel natural products [43]. In this study, six culturable *Bacillus* spp. isolates (*B. cereus* str. S42, *B. tequilensis* str. SV39, *B. subtilis* str. SV41, *B. methylotrophicus* str. SV44, *B. amylolequifaciens* subsp. *plantarum* str. SV65 and *B. mojavensis* str. SV104), isolated from healthy stems of wild Solanaceae species (*N. glauca*, *D. metel*, *S. nigrum* and *S. elaeagnifolium*), were evaluated for their antifungal potential towards FOL and they have been explored as potential sources of bioactive metabolites.

Tested using the dual culture method, *Bacillus* spp. exhibited a strong antifungal activity against FOL that may be attributed to their diffusible active metabolites. Previous studies have shown that endophytic *Bacillus* spp., isolated from *Salvia miltiorrhiza* [24] and/or *Pinus taeda* L. [44] may be useful as BCAs and potential sources of bioactive molecules. *Bacillus* species were also reported to produce non-volatile antifungal metabolites [6,45] and/or volatile compounds [41]. In fact, tested using the sealed plate method, all *Bacillus* spp. isolates tested in the current study had significantly reduced FOL growth compared to the untreated control. This method of confrontation revealed the presence of antifungal volatile metabolites active against FOL. The diffusible metabolites from *Bacillus* spp., tested in the present study, caused greater growth inhibition of FOL (38.7 to 46.5%) than the volatile compounds (18.2 to 21.6%). However, Chaurasia et al. [35] found that the inhibitory effect attributed to volatiles was greater than that induced by diffusible compounds where *B. subtilis* was used as an antagonistic agent against *F. oxysporum*, *Alternaria alternata*, *Cladosporium oxysporum*, *Paecilomyces lilacinus*, *Paecilomyces variotii*, and *Pythium afertile*.

In order to optimize the production of antifungal metabolites, cell-free filtrates of the six *Bacillus* spp. isolates were extracted from cultures grown at different times of incubation. Results showed a significant inhibition of the pathogen using filtrates issued from 2-, 3-, 4-, and 7-day-old cultures with a maximum (from 31.1 to 59.5%) recorded after 4 days of incubation compared to the untreated controls. Romero et al. [38] found that the antifungal activity of the cell-free cultures of *B. subtilis* was detected at the transition phase between exponential and stationary phase of growth. This activity increased progressively during the later and reached its highest levels after 4 to 5 days of culture when bacterial populations were composed mainly by spores. Our findings clearly demonstrated the possibility of using *B. cereus* str. S42, *B. tequilensis* str. SV39, *B. subtilis* str. SV41, *B. methylotrophicus* str. SV44, *B. amyloliquefaciens* subsp. *plantarum* str. SV65 and *B. mojavensis* str. SV104 as sources of biologically active natural products with an optimum of metabolites production recorded at 4 days of incubation. Other studies also reported secondary metabolites from endophytic *Bacillus* spp. with inhibitory effects against *Fusarium* spp. and other plant pathogens [16,44].

The secondary metabolites produced by *Bacillus* species exhibited diverse chemical structures and biological activities [47]. All organic extracts tested had significantly reduced FOL growth by 37 to 90%, compared to the ethanol controls, and this depending upon the concentrations used. Biologically active metabolites extracted with ethyl acetate, diethyl ether, chloroform, n-hexane, acetone and n-butanol from endophytic *Bacillus* species also inhibited the mycelial growth of several pathogenic fungi and/or bacteria [42,16,17].

The two types of organic extracts from *Bacillus* spp. isolates tested were found to be more effective at the concentration of 5% than at 2.5% (v/v). The highest antifungal potential toward FOL growth (90%) was achieved using the n-butanol extract from *B. amylolequifaciens* subsp. *plantarum* str. SV65 used at 1 mg/mL (w/v). In the same sense, the n-butanol extract from endophytic *B. subtilis* str. ZZ120 (1 mg/mL w/v) led to 61.4% decrease in *F. graminearum* growth [16]. Tested at 2.5% (v/v), chloroform extracts from *B. tequilensis* str. SV39 and *B. cereus* str. S42 reduced pathogen growth by 72.4 and 71.3%, respectively as compared to the ethanol control. Bhoonobtong et al. [37] also demonstrated the antifungal potential of chloroform extracts from an endophtyic *B. amylolequifaciens* str. D25, isolated from medicinal plant, towards *Staphylococccus aureus*. However, chloroform extracts from four strains of *B. subtilis* (*B. subtilis* str. UMAF6614, *B. subtilis* str. UMAF6619, *B. subtilis* str. UMAF6639 and *B. subtilis* str. UMAF8561) were found to be ineffective towards the pathogen *Botrytis cinerea* [38]. Previous studies, using *Bacillus* spp. extracts showed that the major identified chemical substances belonged to the families of aldheydes, ketones, benzenes [47], dimethyl disulfide [48] and phthalic acids [27].

Conclusion

Plant-associated microorganisms, especially endophytic bacteria, represent largely untapped resources of natural products. Six nonpathogenic and culturable *Bacillus* spp. (*B. cereus* str. S42 (KP993206), *B. tequilensis* str. SV39 (KR818070), *B. subtilis* str. SV41 (KR818071), *B. methylotrophicus* str. SV44 (KR818072), *B. amyloliquefaciens* subsp. *plantarum* str. SV65 (KR818073) and *B. mojavensis* str. SV104 (KR818089)), isolated from stems of wild Solanaceae (*N. glauca*, *D. metel*, *S. nigrum* and *S. elaeagnifolium*), were found to be potential sources of non-volatile and/or volatile bioactive metabolites effective against FOL. Chloroform and n-butanol extracts from *Bacillus* spp. exhibited an interesting antifungal potential towards FOL higher than that induced by the two commercial products i.e. Bavistin® (50%, chemical fungicide) and Bactospeine® (16000UI/mg, biopesticide). These endophytic bacterial isolates could be interesting for use as bio-fungicides against FOL.

Testing the antifungal activity of cell-free cultures of these *Bacillus* spp. isolates and their organic extracts *in vivo* may give additional information on their effects on Fusarium wilt suppression and tomato growth promotion. Chemical analyzes of the most effective organic extracts will identify the major compounds.

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