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RESEARCH ARTICLE

In Vitro Antagonistic Activity of Diverse *Bacillus* Species Against *Fusarium culmorum* and *F. solani* Pathogens

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Abstract:

Background:

Fusarium culmorum and *Fusarium solani* are economically important fungal pathogens of many plant species causing significant yield losses worldwide. Frequent uses of fungicides are hazardous to humans and the environment. Therefore, *in vitro* antagonistic activity of diverse *Bacillus* species isolates with biological potential activity to control these both pathogens should be investigated.

Objective:

The objectives were to isolate and identify the *Bacillus* spp., which are potential controls of *F. culmorum* and *F. solani*, and to characterize molecularly, at the species level, those isolates that have potential as biocontroller of the pathogens.

Methods:

The *in vitro* antagonistic potential of 40 *Bacillus* isolates against *F. culmorum* and *F. solani* was evaluated on the basis of fungal growth inhibition on nutrient broth culture. The colony morphology and the 16S rRNA gene sequencing of *Bacillus* spp. were used to identify the isolates.

Results:

Bacillus sp. isolates were identified as *B. atrophaeus, B. subtilis, Paenibacillus polymxa, B. amyloliquefaciens, B. simplex* and *B. tequilensis*. They had significant (P<0.05) antagonistic activities against *F. culmorum* and *F. solani* isolates as compared to the untreated control. The antagonistic effects varied depending on the *Fusarium* sp. The bacterial *B. subtilis* isolates SY116C and SY SY118C provided the most noteworthy result as both strongly inhibited mycelial growth of *F. solani* by 97.2%, while the *B. tequilensis* isolate SY145D was the most effective in the formation of inhibition zones against *F. culmorum* by 75%.

Conclusion:

It is apparent that *Bacillus sp.* isolates play an important role in the inhibition of growth of *F. culmorum* and *F. solani*, and that the *B. subtilis* isolates SY116C and SY118C had the highest biological potential activity against these fungi. These antagonistic effects may be important contributors as a biocontrol approach that could be employed as a part of integrated soil pathogen management system.

Keywords: Antagonistic effect, *Bacillus* species, *Fusarium* species, *In vitro*, Fungal pathogens , Biocontrol approach .

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1. INTRODUCTION

Fusarium culmorum and *Fusarium solani* are important species that cause significant yield losses in many plant species [1, 2]. *Fusarium culmorum* is a soil-borne fungus distributed in cooler temperate regions, and the causal agent of many important diseases in cereals [3, 4]. *F. solani* is known to infect many plant species, causing plant decline, wilting, and large necrotic spots on tap roots [5, 6].

Fungicides are commonly used to control these diseases, but frequent uses of these chemicals are hazardous to humans and the environment. Therefore, the management of the soil-borne pathogens has become one of the major concerns in agriculture and focused on searching and selecting antagonist microorganisms on diverse soil pathogens [7, 8]. However, difficulties in controlling *Fusarium* sp. have stimulated renewed interest in the application of biological control agents, which has provided an effective and environmentally friendly means to control plant diseases. Among the most widely used microbes for biocontrol agents are members of the genus

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Bacillus, that offer advantages over other microorganisms, tolerance to fluctuating pH, temperature and osmotic conditions [9, 10]. Furthermore, *Bacillus* spp. are able to colonize root surfaces, promoting plant growth and causing mycelium lysis of several fungal agents [11, 12].

However, soil-borne fungal pathogens such *Fusarium* sp. have been shown varying metabolic responses, growth patterns and reproductive strategies in response to varying soil microorganisms, therefore, measuring the *in vitro* growth rate of fungi was considered as a simple and reliable method for evaluating the effect of an environmental variable on the growth of fungi, although this does not take into account changes in mycelia density [13].

On the other hand, the 16S ribosomal RNA (16S rRNA) gene has been widely used for the taxonomic classification of bacteria by the detection of sequence differences in the hypervariable regions of the 16S rRNA gene which is present in all bacteria [14, 15].

During a polyphasic experiment, more than 525 bacilli were isolated from different regions of Syria. In the present study, forty of them were taken into the 16S rRNA gene sequence analyses.

The objectives of this current work were to isolate and identify the bacteria which have the potential to control *Fusarium* spp. (*F. culmorum* and *F. solani*) and molecular characterization of species that have the potential for biocontrol of the pathogen.

2. MATERIALS AND METHODS

2.1. Isolates of *Bacillus* sp.

The soil samples were collected from different regions distributed widely from south to the north-west between 33.40°N and 37.17°N and 35.40°E and 42.30°E in Syria. They were taken from 2-3 cm depth of field and were carried to laboratory in sterile polythene bags. They were shaken in 9 ml sterile water for 3 min at 160 rpm. Serial dilution was made from 10^{-3} to 10^{-7} [16] and then 0.2 ml of each dilution was spread onto Nutrient Agar (NA) medium and incubated overnight, the colonies of prospective *Bacillus* sp. were identified according to Wulff et al. [17], and 40 isolates were selected for this study (Table **1**). Six *Bacillus* species, namely, *B. atrophaeus, B. subtilis, P. polymxa, B. amyloliquefaciens, B. simplex* and *B. tequilensis*, were selected for the further *in vitro* study. A pure culture of each *Bacillus* sp. isolate was first grown on NA and incubated for 24h at 30°C.

2.2. Fungal Isolates

Fusarium culmorum and *F. solani* were isolated from infected wheat plants growing in different locations of Syria. The infected wheat stems were cut into small pieces of 1-1.5 cm, surfaces were sterilized with 5% sodium hypochlorite for 5 min, washed in sterile distilled water twice and cultured on PDA (PDA, DIFCO, Detroit, MI. USA) medium amended with 13 mg/l kanamycin sulphate added after autoclaving and incubated for 10 days, at 23 ±1ºC in the dark to allow mycelial growth and sporulation. Species identification was based on the morphological characteristics of single spored isolates as described by Nelson et al. [18, 19]. According to a study [20], the two virulent monosporic isolates of *F. culmorum* and *F. solani* were selected for this study. The cultures were maintained on silica gel at 4 ºC until needed.

Table1. *Bacillus* species used in the study.

Bacillus species	Number of isolates	Morphology
B. atrophaeus	3	Brown-black, opaque, smooth, circular
B. amyloliquefaciens	10	Creamy white with irregular margins
P. polymyxa	2	Milky white, thin often with amoeboid spreading
B. subtilis	20	Fuzzy white, opaque, rough, with jagged edges
B. simplex	1	Cream, gloss, with irregular margins slightly raised
B. tequilensis	4	Yellowish, opaque, smooth, circular

2.3. *In vitro* Activity of *Bacillus* spp. Isolates against *Fusarium* spp.

Bacterial colonies of different size, color and morphology were streaked individually a few times until single colonies of a single type were observed on the NA plates. Then 5 mm diameter disc of each *F. culmorum* and *F. solani* was cut from of an actively growing culture by a sterile cork borer and placed onto the center of above NA plates. Where mycelia disc on Nutrient Agar (NA) medium without bacteria was maintained as control. Every elementary treatment was repeated five times. The mean diameter of pathogen colonies was measured after 4 days of incubation at 25°C and any morphological alteration of colonies, in comparison to the untreated control, was also noted. Damage caused by the bacterium to the fungal mycelium, removed from the confrontation zone of both microorganisms (pathogen and antagonist), was observed under a light microscope, in comparison to untreated controls. The percentage of inhibition of fungal growth was calculated by the following formula proposed by Rabindran and Vidyasekaran [21].

$$I = (C-T)/C \times 100$$

Where; I = Percent inhibition, C = Radial growth of the pathogen in control, and T = Radial growth of the pathogen in treatment.

2.4. *16S rRNA* Gene Sequencing

Selected bacterial 16S rRNA was amplified in full length by PCR using two pairs of primers, BacF (5'-GTGCCTAATACATGCAAGTC-3') and BacR (5'-CTTTACGCCCAATAATTCC-3') [12]. The PCR reaction mix (50 µl) contained 2 µl (50-100 ng) of extracted genomic DNA, 1x reaction buffer (TrisKCl-MgCl2), 2 mM MgCl2, 0.2 mM dNTP, 1 µM of each primer, and Taq polymerase (5U/µl, Fermentas). PCR amplification condition was achieved using

the following parameters: An initial denaturation step at 95°C for 5min followed by a second denaturation step at 95°C for 1min, annealing for 1min at 54°C, an extension at 72°C for 90s, and a final extension step of 72°C for 10min. A total of 30 serial cycles of amplification reaction were performed. PCR products were separated on a 1.5% agarose gel and visualized using UV light (302 nm) after staining with ethidium bromide. Prior to sequencing, PCR products were purified with QIAgen gel extraction kit (28704) according to the manufacturer´s recommendations. Sequencing was carried out on a Genetic Analyzer (ABI 310, Perklin-elmer, Applied Biosystems, USA). The 16S rRNA sequences were compared with the known sequences using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih. gov).

2.5. Statistical Analyses

All experiments were performed in triplicate with ten Petri dishes per replicate, for each bacterium-fungus *in vitro* evaluation, using a completely randomized design. An F-test was used to determine if the two runs of each experiment were homogeneous and if the data could be pooled. The homogeneity of variance test indicated that the data from both runs of each experiment could be pooled, and thus all further analyses were conducted on pooled data. Data were analyzed using analysis of variance (ANOVA) and means were separated by Tukey's test ($P \leq 0.05$).

3. RESULTS AND DISCUSSION

In this present work, the antagonistic potential of the *Bacillus sp.* isolates was concluded and validated by restriction of the *F. culmorum* and *F. solani* pathogens growth and showed zone of inhibition towards the antagonist as shown in photo-plate of NA culture plate assay compared with the control (Fig. **1**).

On the other hand, PCR amplification with specific primers Bac yielded single DNA fragments of ~ 545 bp, present in all *Bacillus sp.* isolates (Fig. **2**). On the basis of 16S rRNA gene sequencing, *Bacillus* sp. isolates are identified as *B. atrophaeus, B. subtilis, P. polymxa, B. amyloliquefaciens, B. simplex* and *B. tequilensis* as their 16S rRNA gene sequences displayed similarities ≤ 98% to their closely related type strains (Table **2**). Species belonging to the *Paenibacillus* genus were previously re-classified under the genus *Bacillus*, based on morphological characteristics. However, the PCR probe tests suggested that a group of eleven species should be considered a new genus, *Paenibacillus*, of which *P. polymyxa* is the type strain [22]. The nucleotide sequences were deposited in GenBank under accession numbers MT159352 to MT159391 (Table **3**).

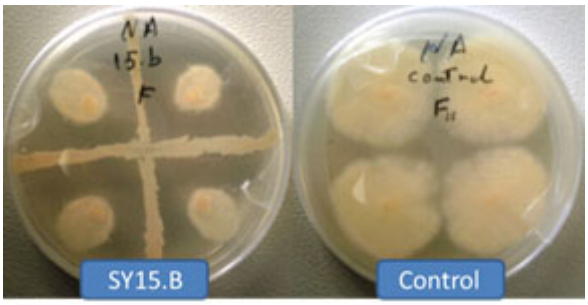

Fig. (1). *Bacillus subtilis* SY15B showing the zone of inhibition in the NA culture plate assay.

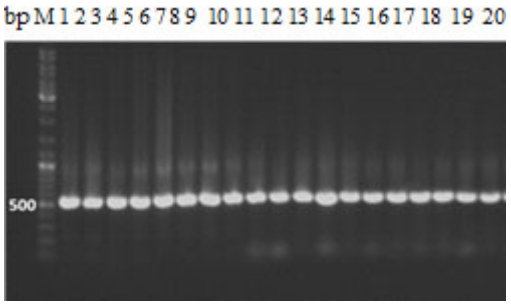

Fig. (2). Agarose gel electrophoresis of 16S rRNA of some *Bacillus sp.* isolates used in the study. M represents the 100-bp DNA marker (*Hin*fI; MBI Fermentas, York, UK).

Table 2. 16S rRNA gene sequence similarity between *Bacillus sp.* used in this study and the microorganisms strains at NCBI.

Bacillus species	Microorganisms (NCBI)	16S rRNA gene sequence similarity (%)
B. atrophaeus	*B. atrophaeus, ATCC 49337*	96%
B. simplex	*B. simplex JP44SK12 (JX144702)*	97%
B. subtilis	*B. subtilis subsp. Spizizenii JP44SK23 (JX144713)*	99%
P. polymxa	*B. paenibacillus macqariensis subsp. Defensor (AB360546)*	98%
B. amyloliquefaciens	*B. amyloliquefaciens (AF478077)*	99%
B. tequilensis	*B. Bacillus tequilensis (KT760402)*	98%

Table 3. *Bacillus* isolates showing antagonistic activity against *Fusarium culmorum* .

No.	Isolates	Zone of Inhibition (%)*	Antifungal activity	GeneBank
				accession number
	B. atroplaeus			
1	SY15B	45h*	+	MT159352
2	SY199A	46h	+	MT159353
3	SY63E	56.7e	+	MT159354
	B. subtilis			
4	SY35A	55ef	+	MT159355
5	Sy41B	53f	+	MT159356
6	SY44A	50g	+	MT159357
7	SY60A	47gh	+	MT159358
8	SY73B	38i	+	MT159359
9	SY113C	60d	++	MT159360
10	SY116C	58.3d	+	MT159361
11	SY118C	53.3f	+	MT159362
12	SY124B	45h	+	MT159363
13	SY130D	46h	+	MT159364
14	SY132E	60d	++	MT159365
15	SY133	63c	++	MT159366
17	SY132C	63.3c	++	MT159367
19	SY134D	61d	++	MT159368
20	SY135D	57.7e	+	MT159369
21	SY139D	36.7i	+	MT159370
22	SY151C	48.3g	+	MT159371
23	SY160C	66.7cd	++	MT159372
24	SY168C	66.7cd	++	MT159373
25	SY190E	43.3hi	+	MT159374
	Paenibacillus polymyxa			
24	SY53C	60d	++	MT159375
25	SY55B	70b	++	MT159376
	B. amyloliquefaciens			
26	SY82C	46h	+	MT159377
27	SY96C	66.7cd	++	MT159378
28	SY96E	61.7d	++	MT159379
29	SY123A	53.3f	+	MT159380
30	SY128B	63c	++	MT159381
31	SY134C	56.7e	+	MT159382
32	SY159D	61.7c	++	MT159383
33	SY177C	53.3f	+	MT159384
34	SY190D	51.7fg	+	MT159385
35	SY200D	57.7e	+	MT159386
	B. tequilensis			
36	SY69A	46h	+	MT159387

(Table 3) contd.....

37	SY145D	75a	+++	MT159388
38	SY149C	71.7ab	++	MT159389
39	SY150D	55ef	+	MT159390
	B. simplex			
40	SY198B	10k	+	MT159391
LSD	0.05			

Zone of Inhibition = (Radial growth of the pathogen in control - Radial growth of pathogen in treatment) / C 100
Weak inhibition: + (Fungal growth was slightly inhibited by bacteria)
Average inhibition: ++ (Loosely arranged mycelial growth over the bacterial zone)
Strong inhibition: +++ (Fungal growth was completely inhibited before the bacterial zone)
*Values followed by different letters are significantly different at P<0.05 according to ANOVA test

The data showed that *Bacillus* sp. isolates had a significant (P<0.05) antagonistic activity against both *F. culmorum* and *F. solani* where the percentage of radial growth inhibition of the fungi colonies significantly decreased on NA medium as compared to the untreated controls (Tables **3** and **4**). In addition, the mean colony diameter of *Fusarium* sp., noted after 4 days of incubation at 25°C, depends upon pathogens tested and treatments realized, which is in agreement with a previous study [23]. However, the antagonistic effects of the bacterial isolates varied depending on the *Fusarium* spp. *B. subtilis* isolates SY116C and SY SY118C inhibited the growth of *F. solani* by 97.2% while the *B. tequilensis* isolate SY145D was the most effective in the formation of inhibition zones against of *F. culmorum* by 75% (Tables **3** and **4**). *Bacillus subtilis* isolates showed similar antagonism against *Rhizoctonia solani*, *Helminthosporium* spp., *Alternaria* spp. and *Fusarium oxysporum* [24], and *B. tequilensis* isolate SY145D showed similar antagonism with *B. tequilensis* GYLH001 that had a potential antagonism towards *Magnaporthe oryzae* of rice [25]

Table 4. *Bacillus* isolates showing antagonistic activity against *Fusarium solani*.

No.	Isolates	Zone of Inhibition (%)*	Antifungal activity
	B. atroplaeus		
1	SY15B	76.8d*	+++
2	SY199A	73.9de	++
3	SY63E	57.8gh	+
	B. subtilis		
4	SY35A	75.4de	+++
5	Sy41B	87c	+++
6	SY44A	76.8d	+++
7	SY60A	100a	+++
8	SY73B	65.2fg	++
9	SY113C	87.1c	+++
10	SY116C	97.2a	+++
11	SY118C	97.2a	+++
12	SY124B	100a	+++
13	SY130D	81.2cd	+++
14	SY132E	78d	+++
15	SY133	76.9d	+++
17	SY132C	76.4d	+++
19	SY134D	68f	++
20	SY135D	77d	+++
21	SY139D	76.2d	+++
22	SY151C	50h	+
23	SY160C	71.2e	++
24	SY168C	88.4c	+++
25	SY190E	71.5e	++
	Paenibacillus polymyxa		
24	SY53C	27i	+
25	SY55B	33.6i	+
	B. amyloliquefaciens		
26	SY82C	87c	+++
27	SY96C	88.4c	+++
28	SY96E	94.5b	+++

(Table 4) contd.....

29	SY123A	84cd	+++
30	SY128B	70.2e	++
31	SY134C	71.2e	++
32	SY159D	62g	++
33	SY177C	73de	++
34	SY190D	69.3f	++
35	SY200D	74.5de	++
	B. tequilensis		
36	SY69A	73.9de	++
37	SY145D	72.4e	++
38	SY149C	85.3cd	+++
39	SY150D	75.1de	++
	B. simplex		
40	SY198B	10k	+
LSD	0.05		

Zone of Inhibition = (Radial growth of the pathogen in control - Radial growth of pathogen in treatment) / C ×100
Weak inhibition: + (Fungal growth was slightly inhibited by bacteria)
Average inhibition: ++ (Loosely arranged mycelial growth over the bacterial zone)
Strong inhibition: +++ (Fungal growth was completely inhibited before the bacterial zone)
*Values followed by different letters are significantly different at P<0.05 according to ANOVA test

It is well known that *B. subtilis* strains produce a broad spectrum of antimicrobial compounds, including predominantly peptides as well as a couple of non-peptidic compounds such as polypeptides, an aminosugar, and a phospholipid [26], and their highly antifungal effects (97.2%) on both *F. culmorum* and *F. solani* in this study, which might be attributed to one or more antifungal compounds produced by this biocontrol agent. However, the observed mycelial growth inhibition and lysis formation among the colonies of the both *Fusarium* pathogens might be due to the effect of the bacterial diffusible inhibitory antibiosis substances, which could have suppressed and restricted the growth of the pathogen, which can be confirmed by the fact that most *Bacillus* spp. have an ability to produce several antibiotics such as bacillomycin, fengycin, mycosubtilin and zwittermicin, which effectively suppress the growth of pathogens under *in vitro* and/or *in situ* conditions [27 - 29]. This might explain the formation of inhibition zones between the bacterial and the *F. culmorum* and *F. solani* isolates shown in this study. Our results are similar to those reported in a previous study [29, 30], which showed high capacity of some strains of *Bacillus sp.* of the same species to inhibit the growth of several phytopathogic fungi; this effect was attributed to the production and secretion of antifungal compounds and antibiotics belonging to the family of iturins and subtilins, that act on the fungi's cell wall [30]. Hence the most likely explanation for the growth reduction of *F. culmorum* and *F. solani* by *Bacillus sp.* is that antifungal activity is increased by co-culturing of different bacterial species.

CONCLUSION

Collectively, this work illustrates that two *B. subtilis* isolates (SY116C and SY SY118C) provided the most noteworthy result as both strongly inhibited mycelial growth of *F. solani* by 97.2%, and that one *B. tequilensis* isolate (SY145D) had a potent antagonistic activity of *F. culmorum* by 75%. These *B. subtilis* and *B. tequilensis* isolates, as potential biocontrol agents, may provide an effective strategy to combat plant pathogens. Field studies should be undertaken to confirm the effectiveness of the isolates under natural field conditions as a component of integrated disease management.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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