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Antagonistic and Inhibitory Effect of *Bacillus subtilis* Against Certain Plant Pathogenic Fungi, I

[1]S.M. Matar, [2]S.A. El-Kazzaz, [2]E.E. Wagih, [3]A.I. El-Diwany,
[1]H.E. Moustafa, [1]G.A. Abo-Zaid, [4]H.E. Abd-Elsalam and [5]E.E. Hafez
[1]Department of Bioprocess Development,
Mubarak City for Scientific Research and Technology Applications, New Borg El-Arab, Alexandria, Egypt
[2]Department of Plant Pathology, College of Agriculture, University of Alexandria, Egypt
[3]National Research Center, Dokki, Cairo, Egypt
[4]Department of Environmental Biotechnology,
[5]Department of Plant Molecular Pathology, Arid Lands Research Institute,
Mubarak City for Scientific Research and Technology Applications, New Borg El-Arab, Alexandria, Egypt

Abstract: The antagonistic and inhibitory activity of fourteen *Bacillus subtilis* isolates (B1 to B14) obtained from different Egyptian sites, were tested against six fungal isolates belonging to four different genera, *Rhizoctonia solani*, *Helminthosporium* spp., *Alternaria* spp. and *Fusarium oxysporum*. Cultural, morphological and physiological characteristics of these isolates were found to be identical to *Bacillus subtilis*. When the fourteen *B. subtilis* isolates were tested as biological control agents for their antagonistic effect on the *in vitro* growth of the fungal isolates, four *B. subtilis* isolates B1, B4, B7, B8 had more antagonistic effect on all fungal isolates. Supernatant of *B. subtilis* isolate B7 had antagonistic effect on 6 fungal isolates but it was more effective on *Helminthosporium* spp., *Alternaria* spp. and *F. oxysporum*. *B. subtilis* as well as, isolate B7 showed effectiveness in reducing disease incidence and severity levels of tomato plants when added to the *F. oxysporum* and *R. solani*-infested soil. Also, it stimulated the growth of tomato plants compared to the other. HPLC analysis of the HCl precipitate of *B. subtilis* isolate B7 culture supernatant revealed that an identical pattern of five peaks to that of a purified preparation of iturin A was obtained.

Key words: *Bacillus subtilis*, shake-flask, biocontrol, iturin A, HPLC analysis

INTRODUCTION

Bacillus subtilis is a non pathogenic gram positive, rod-shaped and endospore-forming aerobic bacterium which is found in soil and rotting plant material. The bacterium which is considered one of the most studied gram-positive bacteria forms colonies that are dull and may be wrinkled, cream to brown in colour and when grown in broth have a coherent pellicle, usually with a single arrangement (Sneath *et al*., 1986). Of the biological control alternatives to chemical pesticides used for minimizing plant disease incidence and severity, the application of nonpathogenic soil bacteria living in association with plant roots is promising. Treatment with these beneficial organisms has, in many cases, been associated with reduction in the adverse effect of plant diseases in greenhouse and field experiments. These bacteria can antagonize fungal pathogens by competing for niche and nutrients, stimulating the defensive capacities of the host plant and producing low-molecular-weight fungitoxic compounds and extracellular hydrolytic enzymes. The mechanism of antibiosis between *B. subtilis* and other microorganisms has been shown to be a challenging topic. Many *B. subtilis* strains produce a small peptide(s) with a long fatty acid moiety, the so-called lipopeptide antibiotics. Most of these antifungal peptides secreted by *B. subtilis* have a molecular weight of less than 2000 Dalton and are synthesized nonribosomally via a multi-enzyme-catalyzed synthesis (Zuber *et al*., 1993). *B. subtilis* RB14 showed antagonistic effect against *Rhizoctonia solani*, the causal agent of damping-off of tomato seedlings. The percentage of diseased plants with damping-off in the absence of *B. subtilis* RB14 was 85.2%. In the presence of culture broth, cell suspension and centrifuged culture broth of *B. subtilis* RB14, the percentage of diseased plants was 16.7, 27.8 and 11.1%, respectively (Asaka and Shoda, 1996). Three *B. subtilis* isolates (B3, C1 and J7) and one

Corresponding Author: Saleh M. Matar, Department of Bioprocess Development,
Mubarak City for Scientific Research and Technology Applications, New Borg El-Arab,
Alexandria, Egypt Tel: (+203) 4593420 Fax: (+203) 4593423

B. polymyxa isolate (D4) showed distinct antagonism against *B. cinerea*, *Pythium aphanidermatum*, *P. mamillatum* and *P. ultimum*. All supernatant filtrates and autoclaved samples of the antagonists cultivated in NB and TSB significantly reduced the levels of conidia germination of *B. cinerea* compared with controls (Walker *et al*., 1998; Jiang *et al*., 2001) showed that *B. subtilis* was the most effective antagonist against *Peronophythora litchi*. Both the antagonist and its extract were effective in controlling artificially wound-inoculated fruit pathogens, but the use of the extract had better effect than that of the antagonist. Chitarra *et al*. (2003) found that the supernatant fluid of *B. subtilis* YM 10-20 inhibited germination of *Penicillium roqueforti* conidiospores. The germinating efficiency of *P. roqueforti* spores after 8 h of incubation in the absence of *B. subtilis* YM 10-20 supernatant fluid was 84%. In the presence of 10, 25 and 50% of supernatant fluid, the percentage of germination decreased to 7, 1 and 0%, respectively. Bais *et al*. (2004) used a wild-type *B. subtilis* 6051 as a biocontrol agent to protect Arabidopsis roots from the infection of *Pseudomonas syringae* pv *tomato* DC3000 *in vitro* and in soil. Similarly, *B. subtilis* BS 107 showed activity *in vitro* and *in vivo* against *Erwinia carotovora* sub spp. *atroseptica* and *E. carotovora* sub spp. *carotovora*, the causal agents of potato black leg and tuber soft rot, respectively (Sharga and Lyon, 1998). Cavaglieri *et al*. (2005) showed that all *Bacillus* strains isolated significantly inhibited *F. verticillioides* growth. *Bacillus* spp. 3 and *B. subtilis* CE1 produced the greatest antifungal activity, 78 and 60%, respectively. Only *B. subtilis* CE1 and *Bacillus* spp. 86, were able to reduce toxin (fumonisin) production, 50 and 29%, respectively. *B. subtilis* CE1 showed the most consistent *F. verticillioides* inhibition of both growth and fumonisin production *in vitro*. *B. subtilis* CE1 at 10^8 and 10^7 colony forming unit (CFU) per milliliter inocula was able to reduce rhizoplane and endorhizosphere colonization of *F. verticillioides* in greenhouse trials. A *Bacillus* strain PY-1 isolated from the vascular bundle of cotton which was identified using biochemical, physiological and 16S rDNA sequence analysis as *Bacillus subtilis* showed strong ability against many common plant fungal pathogens *in vitro* (Gong *et al*., 2006).

The objective of this study was for isolation and physiological identification of bacterial isolates which have an antagonistic effect against certain plant pathogenic fungi.

MATERIALS AND METHODS

Bacterial and fungal isolates: Fourteen *B. subtilis* isolates were isolated in 2002-2003 from different locations in Egypt (El-Malaha, El-Amria, El-Nobaria, Abo-Homos and El-Sharkia) and identified according to the morphological, biochemical and physiological tests recommended by Sneath *et al*. (1986) and Collee *et al*. (1996). Six fungal isolates belonging to four different genera were isolated from different hosts and area in Egypt, *R. solani*, *Helminthosporium* spp., *Alternaria* spp. and *F. oxysporum*.

Antagonistic effect of *B. subtilis* isolates (*in vitro*): *B. subtilis* isolates were used in *in vitro* sensitivity experiments against several fungal isolates, these fungi were isolated from different hosts including *R. solani* from cotton, strawberry and potato; *Helminthosporium* spp., *Alternaria* spp. and *F. oxysporum* from tomato. Potato dextrose agar plates were inoculated with antagonistic isolate of *B. subtilis* as a streak line with a loop-full of 2 days-old culture, incubated for 48 h prior inoculation by the tested fungus. Mycelial disc (5 mm in diameter) of an actively growing culture of the tested fungus was placed in the center at a constant distance opposite to the other edge of the Petri plate and incubated at 30°C for 3-7 days (Toure *et al*., 2004). Inhibition zones (the distance between the edge of antagonistic bacterial growth and the edge of tested fungal isolates) were measured. All experiments were carried out with four replicates for each fungus. The data obtained were statistically analyzed using the Statistical Analysis System (SAS).

Cultivation in shake-flask: Growth of fourteen *B. subtilis* culture was conducted in a shake flask under constant control conditions of temperature, pH and agitation. Bacterial colonies of *B. subtilis* isolates were inoculated into nutrient broth medium (peptone, 5 g L^{-1} and beef extract, 3 g L^{-1}) incubated overnight (16 h) at 30°C with constant shaking at 200 rpm. For antibiotic production, 1 mL of each culture was transferred into a 500 mL Erlenmeyer flask containing 99 mL of Number 3 medium of which each 1 L contained 10 g peptone, 10 g glucose, 1 g KH_2PO_4 and 0.5 g $MgSO_4.7H_2O$ in distilled water and a pH of 6.8 (Asaka and Shoda, 1996). Bacteria were incubated overnight at 30°C with constant shaking at 200 rpm. During the time of cultivation, several samples of culture were taken and cell number was determined by measuring the optical density at 550 nm for obtaining the optimized maximum mass weight and maximum yield of antifungal.

The inhibitory effect of cell free supernatant of *B. subtilis* isolates (*in vitro*): Cell free supernatants of *B. subtilis* liquid cultures of the investigated isolates were tested for its ability to inhibit the growth of the fungus to be investigated. *B. subtilis* isolates were incubated overnight at 30°C with constant shaking at

200 rpm. After 24 h, cultures were removed and centrifuged at 5590 x g for 30 min. Cell free supernatant was filter (0.22 μm) sterilized and tested for antifungal activity. Two wells (5 mm in diameter) were made in each plate of PDA medium using a sterilized cork borer. Subsequently, the wells were filled with 200 μL of supernatant. Mycelial plug (5 mm in diameter) of an actively growing culture of the fungus to be tested was placed in the center of each plate. Plates were incubated for 3-7 days at 30°C, after which, the diameter of the inhibition zone (the distance between the edge of wells and the edge of the tested fungal isolates) was measured, in case of *R. solani* the vertical distance was measured between the edge of plate and the edge of tested fungal isolates). All experiments were carried out with three replicates per fungus (Chitarra *et al*., 2003). The data obtained were statistically analyzed using the Statistical Analysis System (SAS).

Antagonistic effect of *B. subtilis* isolate B7 (*in vivo*): To prepare *F. oxysporum* and *R. solani* (R1) inocula for inoculating soil, 50 mL of sterile PD broth medium in 500 mL Erlenmeyer flasks was inoculated with 5 mm plugs taken from a PDA Petri plate cultures (3-7 day-old). Flasks were incubated without shaking in the dark at 30°C for 1 week. Mycelial mats, on the surface of the medium were then homogenized at 894 g for 2 min in sterile water and mixed with the soil at the ratio of 20 mL of the homogenized mycelial mats to one pot, 5 days before planting tomato.

For inoculation of *B. subtilis* isolate B7 into the soil, the bacterium was inoculated into nutrient broth medium and cultured overnight at 30°C with constant shaking at 200 rpm and then 1 mL of culture broth was transferred to 500 mL Erlenmeyer flask containing 99 mL of Number 3 medium (Asaka and Shoda, 1996) and the culture leaved overnight at 30°C with constant shaking at 200 rpm. Liquid cultures were centrifuged at 894 g for 10 min at 4°C and the pelleted cells were washed in a saline solution (0.85% NaCl, pH 7.0) and then centrifuged again under the same conditions. Washed cells were resuspended in sterile distilled water and 20 mL of the cell suspension was added to each pot 3 days before planting tomato. For each treatment, 4 pots were prepared and 3 plants were transplanted in each. Fresh and dry weight of both shoots and roots were measured at the same time (Asaka and Shoda, 1996). The data obtained were statistically analyzed using the Statistical Analysis System (SAS).

HPLC analysis

Isolation of antifungal compound(s) from *B. subtilis*: Colonies of *B. subtilis* were inoculated each into nutrient broth medium and cultured overnight (16 h) at 30°C with constant shaking at 200 rpm. An aliquot of 1 mL of the culture was transferred into 500 mL Erlenmeyer flask containing 99 mL of number 3 medium and cultured overnight at 30°C with constant shaking at 200 rpm. After 24 h, culture was removed and centrifuged at 894 g for 15 min at 20°C. The supernatant was filtered through a sterile filter (0.22 μm). Subsequently, the pH of the supernatant was adjusted to 2 with concentrated HCl. After centrifugation at 894 g for 10 min at 20°C, the precipitate was collected and dissolved in a mixture of methanol:water (50:50 v/v) pH 8, filtered through 0.22 μm membrane filter and then stored at -20°C until analyzed (Chitarra *et al*., 2003).

Identification of antifungal compound(s) by HPLC: HPLC analysis was performed by injecting 50 μL of the extracted material of *B. subtilis* on a zorbax eclipse XDB-C18 column, 4.6×150 mm, 5 μm (Agilent technologies, USA) and monitoring at 214 nm. Elution at a rate of 0.9 $cm^3 min^{-1}$ was performed in a linear gradient pattern with a mixture of methanol:water (50:50 v/v) during the period 0-20 min, (80:20 v/v) during 20-60 min, (100:0 v/v) during 60-65 min and again (50:50 v/v) during 65-75 min (Chitarra *et al*., 2003).

RESULTS

When the fourteen *B. subtilis* isolates were tested as biological control agents for their antagonistic effect on the *in vitro* growth of the fungal isolates reported here, the following results were obtained:

B. subtilis isolates B8, B1, B7 and B4 which showed no significant differences among each other, had more antagonistic effect on *R. solani* isolated from cotton (R1) than the other *B. subtilis* isolates (Table 1, Fig. 1).

Isolate B1, compared to other isolates, had more antagonistic effect on *R. solani* isolated from strawberry (R2) and potato (R3) and on *Helminthosporium* spp. but not on *F. oxysporum* (Table 1).

By contrast, isolate B1 and B7 whose antagonistic effect did not significantly differ, isolate B7 antagonized the *in vitro* growth of *Alternaria* spp. isolated from tomato and its antagonistic effect exceeded those exhibited by the other isolates (Table 1).

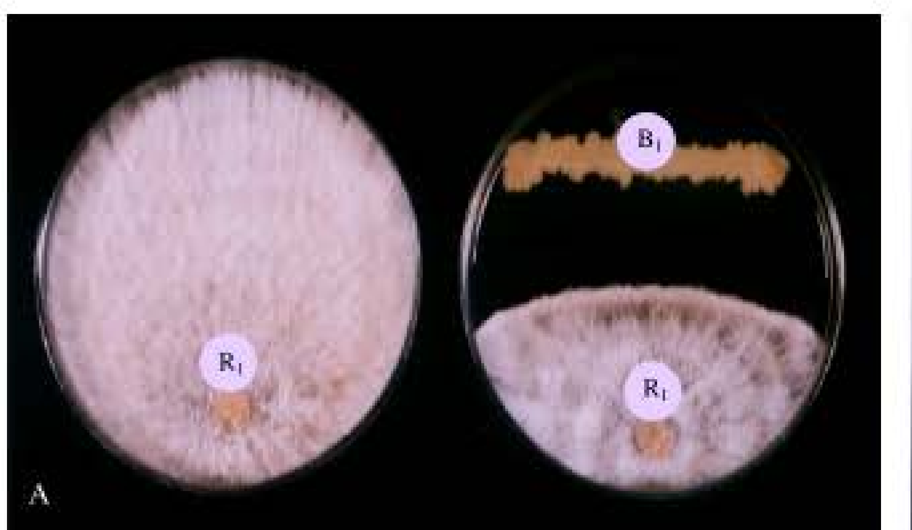
Three *B. subtilis* isolates, B8, B7 and B11 whose antagonistic effect did not significantly differ, were the most effective isolates, followed by B4 and B1 in inhibiting the mycelial growth of *F. oxysporum* isolated from tomato (Table 1, Fig. 1).

When batch cultivation in shake flasks was conducted by inoculating a flask containing a fresh

Table 1: Antagonistic effect of *Bacillus subtilis* isolates against the *in vitro* growth of certain phytopathogenic fungi, *Rhizoctonia solani*, *Alternaria* spp., *Helminthosporium* spp. and *Fusarium oxysporum*

	Vertical distance of inhibition area (cm)					
B. subtilis isolate	*R. solani* (cotton) (R1)	*R. solani* (strawberry) (R2)	*R. solani* (potato) (R3)	*Alternaria* spp. (A)	*Helminthosporium* spp. (H)	*F. oxysporum* (F)
B1	2.250^{a}	2.675^{a}	2.125^{a}	2.425^{ab}	2.525^{a}	0.575^{b}
B2	1.525^{bc}	2.150^{bc}	1.600$_{ef}$	1.100^{e}	1.450^{def}	0.000^{c}
B3	1.350^{c}	1.575^{ef}	1.500^{fg}	1.100^{e}	1.275^{fgh}	0.000^{c}
B4	2.125^{a}	2.025^{bc}	2.050^{ab}	2.150^{bc}	2.200^{bc}	0.625^{b}
B5	0.950^{d}	0.700^{g}	1.225^{h}	1.025^{ef}	1.425^{defg}	0.000^{c}
B6	1.675^{b}	1.650^{def}	1.525^{fg}	1.425^{d}	1.575^{d}	0.000^{c}
B7	2.150^{a}	2.050^{bc}	1.825^{cd}	2.650^{a}	2.375^{ab}	1.225^{a}
B8	2.325^{a}	2.300^{b}	1.900^{bc}	2.150^{bc}	2.100^{c}	1.325^{a}
B9	0.850^{d}	1.300^{f}	1.325^{gh}	0.775^{f}	1.225^{h}	0.000^{c}
B10	1.400^{bc}	2.175^{bc}	1.525^{gf}	2.000^{c}	1.300^{efgh}	0.000^{c}
B11	1.325^{c}	1.975^{bcd}	1.625^{def}	0.000^{g}	1.250^{gh}	1.225^{a}
B12	1.575^{bc}	2.075^{bc}	1.750^{cde}	0.875^{ef}	1.200^{h}	0.000^{c}
B13	1.325^{c}	1.900^{cde}	1.225^{h}	0.875^{ef}	1.225^{h}	0.000^{c}
B14	1.575^{bc}	2.075^{bc}	1.650^{def}	2.000^{c}	1.475^{de}	0.000^{c}
LSD 0.05	0.3244	0.3738	0.2146	0.3126	0.1945	0.1388

Mean values with the same letter(s) are not significantly different



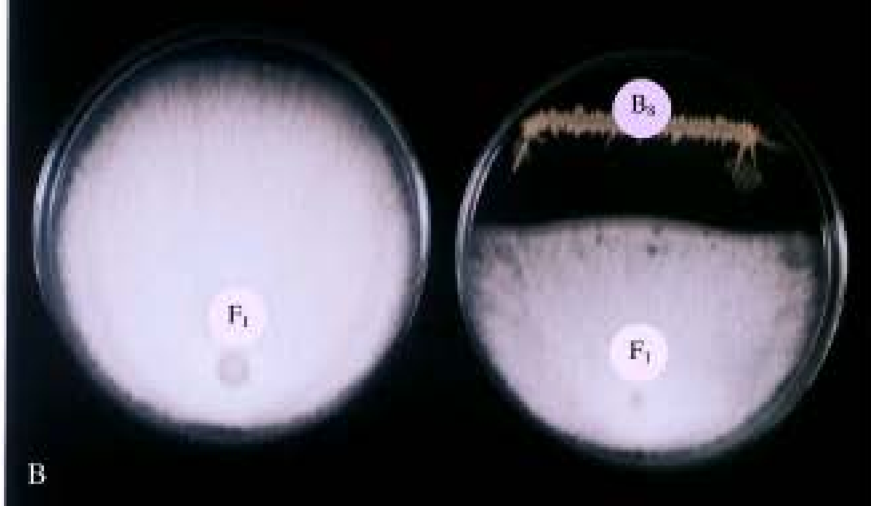


Fig. 1: Antagonistic effect of A, *Bacillus subtilis*, B1 and B, *Bacillus subtilis*, B8 against *Rhizoctonia solani* isolated from cotton (R1) and *Fusarium oxysporum* isolated from tomato, respectively

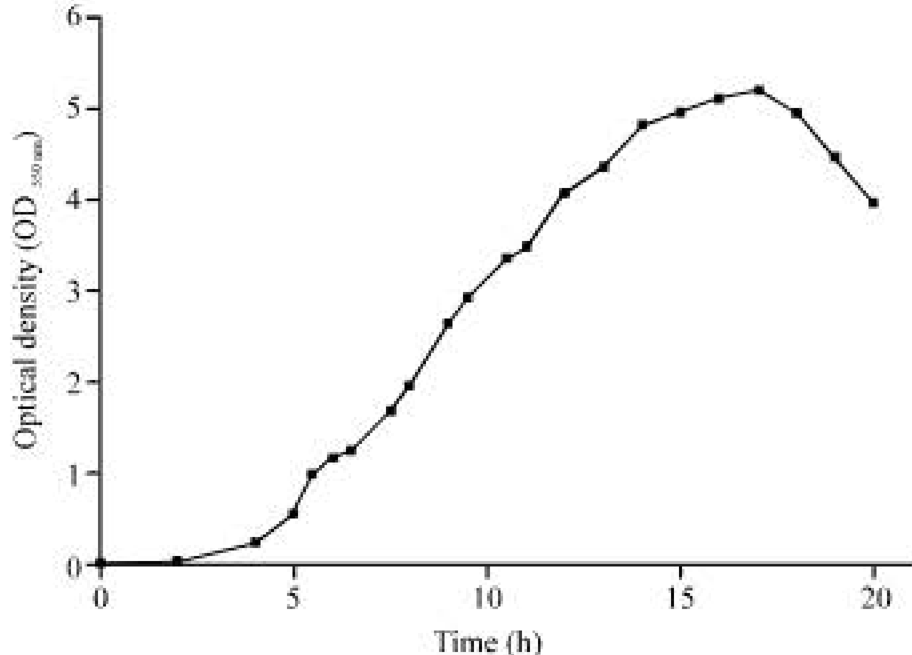


Fig. 2: Growth curve of shake flask cultivation of *Bacillus subtilis* isolate B7, as indicated by optical density as a function of time

medium with a pure culture of each *B. subtilis* isolates, the flask was incubated under agitating at 30°C for 16 h to serve as a source of inoculum to initiate a fresh culture, typical growth curves were obtained (Fig. 2) when the growth was monitored in shake flasks by measuring optical density as a function of time. All curves started with a lag phase which lasted for about 2 h. This phase was followed by the exponential (logarithmic) phase during which optical density increased steadily over time expressing a constant growth rate. The stationary phase started at the end of the exponential phase with a growth rate of zero, after which the culture entered a declining phase.

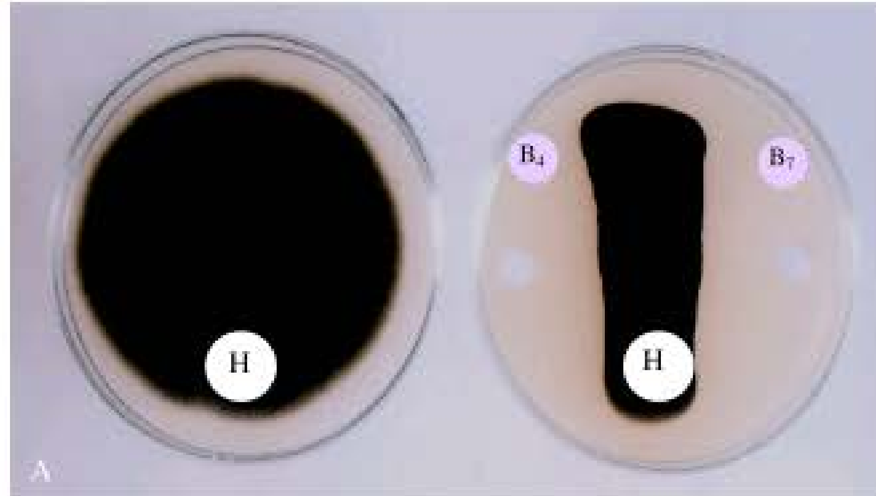
When the experiment was carried out to investigate the effect of cell free supernatant of fourteen *B. subtilis* isolates on the *in vitro* growth of *R. solani* isolated from cotton, strawberry and potato; *Helminthosporium* spp., *Alternaria* spp. and *F. oxysporum* isolated from tomato, results obtained revealed the following:

Supernatant of *B. subtilis* isolates B8, B4 followed by B7, B1 and B10 and lastly B14 inhibited in a descending order, the *in vitro* growth of *R. solani* isolated from cotton

Table 2: The inhibitory effect of cell free supernatant of *Bacillus subtilis* isolates against *in vitro* growth of certain phytopathogenic fungi, *Rhizoctonia solani*, *Alternaria* spp., *Helminthosporium* spp. and *Fusarium oxysporum*

	Vertical distance of inhibition area (cm)					
B. subtilis isolate	*R. solani* (cotton) (R1)	*R. solani* (strawberry) (R2)	*R. solani* (potato) (R3)	*Alternaria* spp. (A)	*Helminthosporium* spp. (H)	*F. oxysporum* (F)
B1	1.266^{bc}	1.233^{b}	0.200^{e}	1.033^{c}	1.366^{b}	0.000^{c}
B2	0.000^{d}	0.000^{d}	0.000^{f}	0.000^{e}	0.000^{d}	0.000^{c}
B3	0.000^{d}	0.000^{d}	0.000^{f}	1.000^{c}	1.700^{a}	0.000^{c}
B4	1.466^{ab}	1.500^{ab}	0.233^{e}	1.266^{abc}	1.833^{a}	0.200^{b}
B5	0.000^{d}	0.000^{d}	0.000^{f}	1.166^{bc}	1.333^{b}	0.000^{c}
B6	0.000^{d}	0.000^{d}	0.000^{f}	0.000^{e}	0.000^{d}	0.000^{c}
B7	1.433^{b}	1.533^{a}	1.233^{b}	1.533^{a}	1.966^{a}	0.400^{a}
B8	1.700^{a}	1.633^{a}	1.500^{a}	0.433^{d}	0.866^{c}	0.000^{c}
B9	0.000^{d}	0.000^{d}	0.000^{f}	0.000^{e}	0.000^{d}	0.000^{c}
B10	1.233^{bc}	1.566^{a}	0.966^{c}	1.366^{ab}	1.866^{a}	0.000^{c}
B11	0.000^{d}	1.233^{b}	0.633^{d}	0.000^{e}	0.000^{d}	0.000^{c}
B12	0.000^{d}	0.000^{d}	0.000^{f}	0.000^{e}	0.000^{d}	0.000^{c}
B13	0.000^{d}	0.833^{c}	0.233^{e}	0.366^{d}	0.800^{c}	0.000^{c}
B14	1.066^{c}	1.566^{a}	0.966^{c}	1.266^{abc}	1.900^{a}	0.000^{c}
LSD 0.05	0.2555	0.2943	0.1612	0.2885	0.2755	0.0000

Mean values with the same letter(s) are not significantly different at $p<0.05$



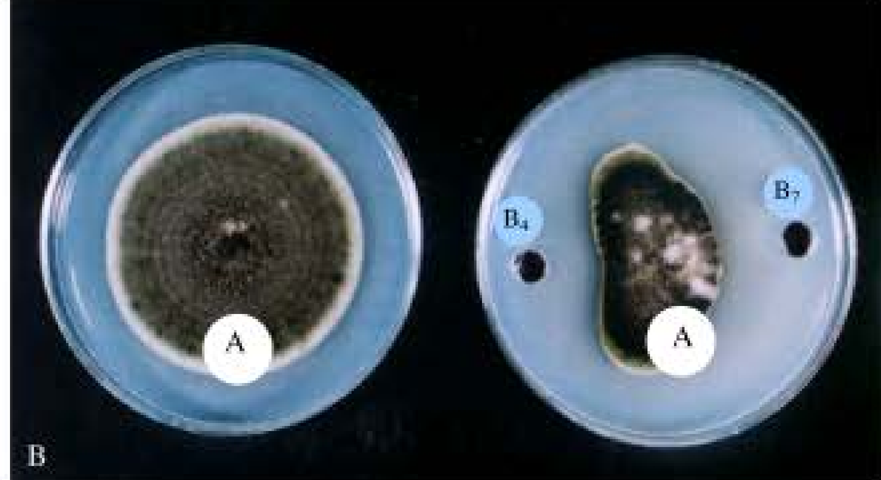


Fig. 3: The inhibitory effect of cell free supernatant of A and B, *Bacillus subtilis*, B4 and B7 against *Helminthosporium* spp. (H) and *Alternaria* spp. isolated from tomato (A), respectively

(R1). Out of those B8 and B4 had the most inhibitory effect, but other isolates seemed to have no observable effect (Table 2).

Supernatant of *B. subtilis* isolates B8, B10, B14, B7 and B4 followed by B1, B11 and lastly B13 had antagonistic effect on *R. solani* isolated from strawberry (R2). However, almost no inhibitory effect was observed by the other isolates (Table 2).

Supernatant of *B. subtilis* isolates B8, B7, B10, B14, B11, B4, B13 and B1 inhibited in a descending order, mycelial growth of *R. solani* isolated from potato (R3) though with significant differences and of which B10 and B14 and similarly B4 and B13 were each equally effective (Table 2).

Supernatant of *B. subtilis* B7, B14, B10, B4 and B3 were more effective on *Helminthosporium* spp. than B1, B5, B8 and B13 (Table 2, Fig. 3).

Supernatant of *B. subtilis* isolates B7, B10, B4 and B14 which showed no significantly differences in their effect, were the most effective, followed by B5, B1, B3, B8 and B13, in inhibiting *Alternaria* spp. isolated from tomato (Table 2, Fig. 3).

Out of all *B. subtilis* isolates, only two isolates, B7 followed by B4, could inhibit mycelial growth of *F. oxysporum* isolated from tomato (Table 2).

B. subtilis isolate B7 was effective in reducing disease incidence and severity levels on tomato plants when added to the *F. oxysporum* and *R. solani*-infested soil 3 days before planting tomato as compared to *F. oxysporum* and *R. solani*-infested soil receiving no bacteria as a control. *B. subtilis* isolate B7 stimulated the growth of tomato plants compared to the other treatments since the fresh and dry shoot weights (6.952 and 0.990 g, respectively) and fresh and dry root weights (1.267 and 0.212 g, respectively) increased significantly. Control treatment inoculated with *F. oxysporum* and *R. solani* without antagonistic agent (*B. subtilis* isolate B7) had significantly less fresh and dry weight of shoot and root (Table 3, Fig. 4, 5).

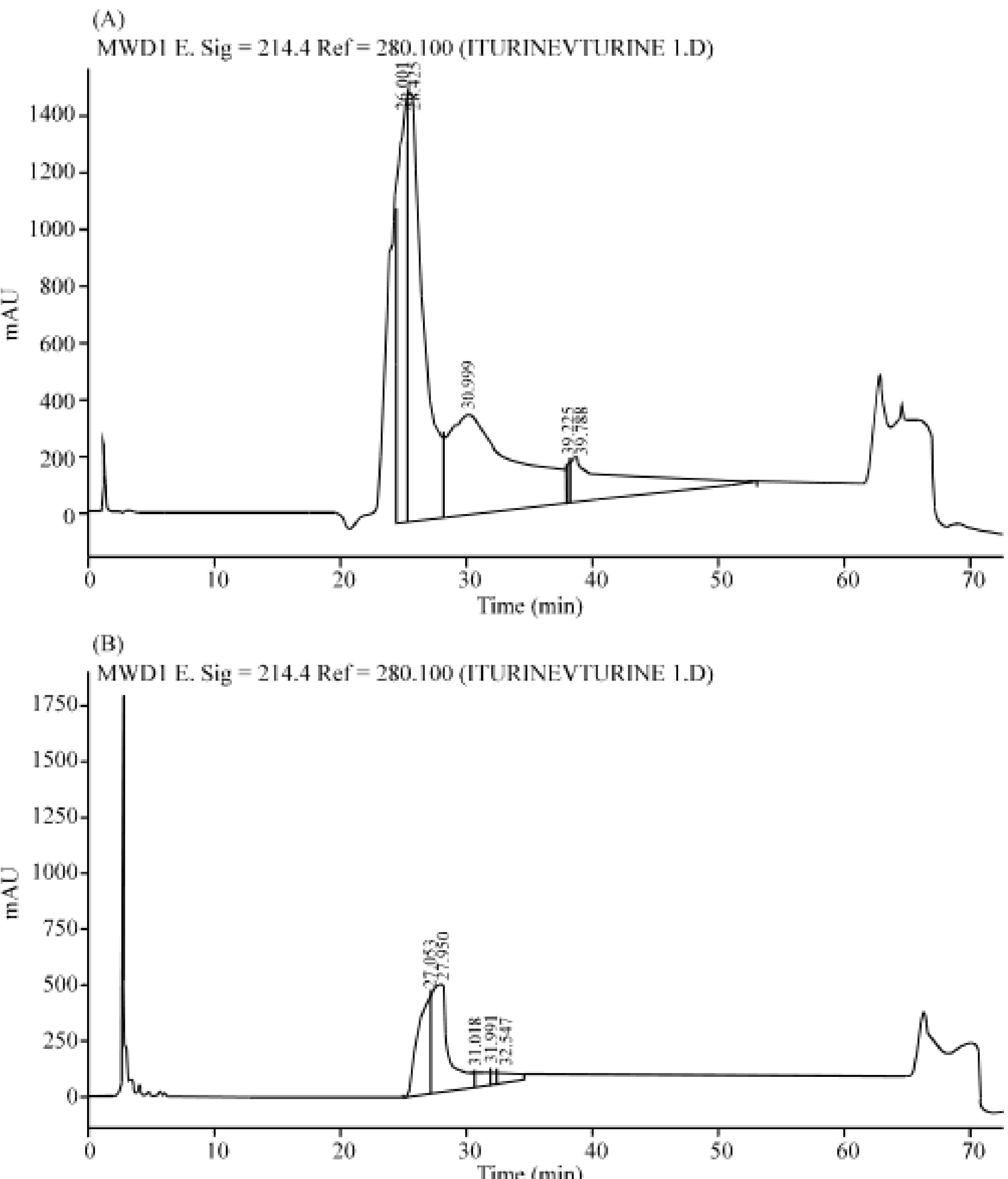


Fig. 6: HPLC analysis of purified iturin A produced by *Bacillus subtilis* (A) and the HCl-precipitate of *Bacillus subtilis* isolate B7 culture supernatant (B)

Table 3: Effect of *Bacillus subtilis* isolate B7 on fresh and dry weight of shoots and roots of tomato plants growing in a soil infested with *Fusarium oxysporum* or *Rhizoctonia solani* (R1)

	Fresh weight g^{-1}		Dry weight g^{-1}	
Treatments*	Shoot	Root	Shoot	Root
C	6.592^{a}	1.212^{a}	0.950^{a}	0.180^{b}
B	6.952^{a}	1.267^{a}	0.990^{a}	0.212^{a}
F	2.050^{c}	0.520^{b}	0.457^{c}	0.082^{d}
B–F	4.467^{b}	1.127^{a}	0.617^{b}	0.137^{c}
R	1.620^{d}	0.147^{c}	0.280^{d}	0.050^{e}
B–R	4.182^{b}	0.742^{b}	0.572^{bc}	0.112^{cd}
LSD 0.05	0.4153	0.2443	0.1315	0.0307

*, C: Control, B: *B. subtilis* isolate B7: F, *F. oxysporum*; B-F, *B. subtilis* isolate B7-*F. oxysporum*; R: *R. solani* (R1); B-R: *B. subtilis* isolate B7-*R. solani*

When HPLC analysis was performed by injecting 50 μL of the HCl precipitate of *B. subtilis* isolate B7 culture supernatant, an identical pattern of five peaks to that of a purified preparation of iturin A (Fig. 6) was obtained.

DISCUSSION

The fourteen *B. subtilis* isolates involved in this study, being obtained from different locations and various plant ecological niches in Egypt may constitute good representative sample of wide diversity. The use of morphological, cultural, physiological, biochemical and molecular characterization of the isolates confirm their identity. Similarly, the phytopathogenic fungi, *Alternaria* spp., *Fusarium oxysporum*, *Helminthosporium* spp. and *Rhizoctonia solani* which were isolated from different hosts including cotton, potato, strawberry and tomato and their various sensitivity to the antagonistic effect of *B. subtilis* increase the reliability of results.

Antagonism is ubiquitous in nature among different species. For a long time, people have been interested in rationally making use of it in the areas of biological control of plant disease. Plant fungal diseases are difficult

to control and can cause huge damage to economic crops. Environmental pollution, caused by abusing chemical biocides, is another serious problem. Using antibiotic producing bacteria to control plant fungal diseases is a popular topic and has extensively been studied (Raaijmakers *et al*., 2002). Compared with chemical biocides, many antibiotics produced by antagonistic strains have the advantage of being easily decomposed in nature, leaving no harmful residues behind. The results, obtained here, of *in vitro* sensitivity of phytopathogenic fungi to antagonistic bacteria revealed that the isolates of *B. subtilis* were suppressive, though with different degrees, to the tested isolates of phytopathogenic fungi, are consistent with those obtained by others (Wagih *et al*., 1989; Gong *et al*., 2006). Interestingly, isolate B1 of *B. subtilis* was found to be the most potent and most common antagonistic isolate to the six isolates of the phytopathogenic fungi tested. Although isolate B7 did not significantly differ in its effect from isolate B1, its antagonistic effect was significantly less than that of isolate B1 when tested against isolate R2 and R3 of *R. solani*. Surprisingly, the inhibitory effect of isolate B1 cell-free culture supernatant did not go along with the antagonistic effect as it was less than that observed with the culture supernatant of other isolates. By contrast, the inhibitory effect of isolate B7 cell-free culture supernatant corresponded with the antagonistic effect it demonstrated against all isolates tested except these of *R. solani*. The discrepancy observed between the results obtained from the antagonistic and those from the inhibitory effect work is likely to be due to the involvement of more than one inhibitory substance of which one or more may be lost during supernatant preparation and manipulation. In spit of this, it might be valid to suggest that the inhibitory effect of the cell-free supernatant of most *B. subtilis* isolates against the tested isolates of phytopathogenic fungi could be due to antifungal compound(s) produced by *B. subtilis* isolates that is likely to be iturin.

The reduction in disease incidence and severity of infection of tomato plants inoculated by *F. oxysporum* and *R. solani* following the use of *B. subtilis* isolate B7 as a soil drench before inoculation demonstrates the potential of using *B. subtilis* as a biological control agent. This could be due to a direct inhibitory effect on the pathogens investigated. But the indirect effect through promoting tomato growth as evidenced by the observed increase in wet and dry weight of shoot and root system can not be ruled out. This study supports that of Marten *et al*. (1999) in which it was shown that *B. subtilis* B2g was able to suppress soil-borne pathogens (e.g., *R. solani*) in the rhizosphere of plants and this strain has been developed as a commercial biological control agent. Similarly supportive results were obtained by Asaka and Shoda (1996) who reported that *B. subtilis* RB14 showed antagonistic effect against damping-off of tomato seedlings caused by *R. solani*.

HPLC analysis of the HCl precipitate from *B. subtilis* isolate B7 showed that this compound is similar to iturin A, five peaks were detected. The same results were obtained by Kita *et al*. (2005); HPLC analysis could detect five peaks comprising iturin A.

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