CHARACTERIZATION OF SOME BIOFILM-FORMING *BACILLUS SUBTILIS* STRAINS AND EVALUATION OF THEIR BIOCONTROL POTENTIAL AGAINST *FUSARIUM CULMORUM*

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SUMMARY

Thirty Iranian native *Bacillus* sp. strains collected from the rhizosphere of various hosts in different regions of the country were studied. 16S rRNA identification and phylogenetic analysis was carried out and secondary metabolites of volatile components, protease activity, biofilm formation and antagonistic activity against *Fusarium culmorum* in greenhouse were investigated for a thorough characterization of the strains. Correlation between biofilm formation and biocontrol capabilities under greenhouse conditions was also studied. PCR amplification and partial sequencing of 16S rRNA showed that all Iranian strains had more than 98% similarity with *Bacillus* sp. strain sequences from GeneBank. Phenotypical characterization allowed their classification as *Bacillus subtilis.* Phylogenetic analysis revealed that the strains clustered into two different phylogenic groups. Results of laboratory and greenhouse experiments showed that volatile and protease production as well as biofilm formation by some strains had a significantly positive correlation with their antagonistic ability. Finally, based on the results from laboratory and greenhouse experiments, the most powerful antagonist strains with high biofilm production were selected for future field experiments.

Key words: *Bacillus subtilis*, biofilm, *Fusarium culmorum*, biocontrol, 16S rRNA, PCR, sequencing.

INTRODUCTION

One of the most important diseases in cereal-growing areas of the world is Fusarium head blight (FHB) caused by a complex of *Fusarium* species including *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae* and *F. sporotrichioides*. FHB is associated with considerable yield losses because of premature senescence, reduced grain filling and their contamination with mycotoxins as the inciting agents produce a range of these products, i.e. trichothecenes, zearalenone, moniliformin and fumonisins, (Müllenborn *et al*., 2008). The most important toxin for human health is the trichothecene deoxynivalenol (DON), which was first identified in late 1979 in the USA. This toxin is a low-molecular weight inhibitor of protein synthesis with cell membrane and hemolytic activity. Feeding of contaminated grains or products of exposed animals has severe long-term consequences, including immunosuppression, neurotoxicity, and nutrient uptake alteration (Lutz *et al*., 2003). The distribution and prevalence of FHB vary in different areas and years and depend on amount of inocula and the environmental conditions, especially temperature and moisture in the period from heading to soft dough (Doohan *et al*., 2003; Müllenborn *et al*., 2008).

F. culmorum (W. G. Smith) Sacc. is one of the most important agents of FHB in tropical and subtropical regions. Its damage to grain yield is high in areas in which flowering and heading stages are contemporary with warm and humid weather (Etebarian and Wilcoxson, 1993). It causes different seed-borne and soil-borne diseases of seedlings, including brown foot rot, root rot, seedling and head blight on wheat (Kempf and Wolf, 1989). This pathogen is localized in the pericarp, but conidia may be carried externally on the testa. Infection can take place through hypocotyl stomata, from where the fungus progresses to the root, shoot and coleoptiles (Knudsen *et al*., 1995).

The excessive application of pesticides affects soil fertility, the environment and threatens human health. To alleviate these problems, biological control agents, which include microorganisms and microbial products, have recently attracted attention as alternatives to chemicals (Mizumoto *et al*., 2006; Mohammadi Pour *et al*., 2009). Understanding the importance of soil and root microbes, the use of molecular techniques for the study of microorganisms, and the possibility of manipulating their actions, has opened new prospects for biological solutions (Knudsen *et al*., 1995; Johansson *et al*., 2003).

As there is no effective chemical control or suitable resistant cultivars to *F. culmorum*, the use of microbes as biological control agents (BCAs) is of interest. There are

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several examples of BCAs (e.g. antibiotic-producing bacteria, fungi and actinomycetes) showing antagonistic activity against *F. culmorum*, such as. *Trichoderma harzianum* (Kempf and Wolf, 1989), *Streptomyces* sp. (Borghi *et al*., 1990), *Pseudomonas fluorescens* and *Bacillus subtilis* (Da Luz and Da Luz, 1994). An important BCA candidate is *B. subtilis*, a model organism for the study of Gram-positive bacteria (Leclere *et al*., 2005; Mohammadi Pour *et al*., 2009; Ongena *et al*., 2005). *B. subtilis* and other closely related *Bacillus* spp. (*B. megaterium*, *B. cereus*, *B. pumilus* and *B. polymyxa*) have received much attention as potential BCAs because of the production of a wide range of different antimicrobial metabolites, among which the non-ribosomal synthesized cyclic lipopeptide-like surfactin iturin A and fengycin (Stein, 2005), several modified small peptides (Kim and Chung, 2004), volatiles (Fiddaman and Rossall, 1993) and biofilm (Bais *et al*., 2004). These BCAs have been used successfully to control a variety of plant pathogenic fungi and bacteria including *Botrytis cinerea* (Toure *et al*., 2004), *Rhizoctonia solani*, *P. solanacearum* (Shoda and Ano, 1994), *Sclerotinia sclerotiorum* (Souto *et al*., 2004), *Xanthomonas oryzae* (Lin *et al*., 2001), *Aspergillus flavus* and *Colletotrichum gloeosporioides* (Mohammadi Pour *et al*., 2009).

El-Meleigi *et al*. (2007) showed that seed treatment with *Bacillus* spp. reduced the severity of common root rot and increased grain yield up to 102% compared to Vitavax-treated seeds. In fact, *Bacillus* spp. have traits such as high thermal tolerance, rapid growth in liquid cultures and resistant endospore formation (Kim *et al*., 2003) that make them suitable BCAs in the soil.

Biofilms are structured communities of cells adherent to a solid surface and encased in an extracellular polymeric matrix. Advantages of biofilm formation include protection of bacteria from adverse environmental conditions, access to nutrient materials via permeable water channels in mature biofilm and incepting new genetic and colonization characters (Davey and O'Toole, 2000; Morris and Monier, 2003; Branda *et al*., 2005). Biofilm production occurs in response to different signals, i.e. environmental and nutrient factors (O'Toole and Kolter, 1998; Fletcher, 1998). Rhizosphere is one of the most important sites for biofilm production by microorganism communities, because the readily available nutrients secreted by plant roots facilitate their growth (Bais *et al*., 2004). It has been shown that a biofilm and surfactin producing *B. subtilis* strain could control a bacterial pathogen of *Arabidopsis* by comparison with a mutant strain unable to produce these compounds (Bais *et al*., 2004). Several functions known to influence biocontrol activities are also likely to play a role in biofilm formation (Dunn *et al*., 2003).

The aim of this study was the characterization of some Iranian *B. subtilis* strains based on phylogenetic relationships and their biocontrol activity potential against wheat root rot due to *F. culmorum*, as well as the evaluation in laboratory and greenhouse levels of the role of biofilm, volatiles and protease production on the antagonistic activities of bacterial strains.

MATERIALS AND METHODS

Microorganisms and growth conditions. *Bacillus* sp. strains used in this study were isolated according to Mohammadi Pour *et al.* (2009) and Shahrokhi *et al.* (2008), from the rhizosphere of rice, sugar beets and almonds growing in various Iranian locations (Table 1). *B. subtilis* strain DSM No. 3258 (from DSMZ-Germany) was used

Table 1. Bacterial strains used in this study.

Strains	Accession number	Host	Locality	Strains	Accession number	Host	Locality
Bs1	HQ197378	rice	Gilan	Bs17	HQ267751	sugar beet	Hamedan
Bs2	HQ234318	rice	Mazandaran	Bs18	HQ267752	sugar beet	Esfahan
Bs3	HQ234319	almond	Esfahan	Bs19	HQ267753	sugar beet	Tahran
Bs4	HQ234320	rice	Gilan	Bs20	HQ267754	sugar beet	Markazi
Bs5	HQ234321	almond	Esfahan	Bs21	HQ267764	sugar beet	Markazi
Bs6	HQ234322	almond	Zanjan	Bs22	HQ267755	sugar beet	Markazi
Bs7	HQ234323	rice	Gilan	Bs23	HQ267756	sugar beet	Lorestan
Bs8	HQ234324	rice	Gilan	Bs24	HQ267757	sugar beet	Fars
Bs9	HQ234325	rice	Golestan	Bs25	HQ267758	sugar beet	Tehran
Bs10	HQ234326	sugar beet	Gazvin	Bs26	HQ267759	sugar beet	Gazvin
Bs11	HQ234327	sugar beet	Kermanshah	Bs27	HQ267760	sugar beet	Tehran
Bs12	HQ234328	sugar beet	Kermanshah	Bs28	HQ267761	sugar beet	Fars
Bs13	HQ234329	sugar beet	Khozestan	Bs29	HQ267762	sugar beet	Esfahan
Bs14	HQ234330	sugar beet	Tehran	Bs30	HQ267763	sugar beet	Kermanshah
Bs15	HQ234331	sugar beet	Tehran	Bs31	_	DSM No. 3258	Germany
Bs16	HQ234332	sugar beet	Fars				

as a standard. The bacterial strains were routinely grown on nutrient agar (NA) or Luria-Bertani broth (LB) at 37ºC. The *F. culmorum* isolate received from Plant Protection Research Institute of Iran (PPRI), was cultivated on potato dextrose agar (PDA) at 27ºC for routine use and transferred to 4ºC for long time maintenance.

Amplification of 16S rRNA gene. Total DNA was extracted and purified according to Welington *et al.* (2004). The primers fD1: 5'-CCGAATTCGTCGA CAACAGAGTTTGATCCTGGCTCAG-3' and rD1: 5'-CCCGGGATCCAAGCTTAAGGAGGTGAT CCAGCC-3' were used for amplification of 16S rRNA (Weisburg *et al*., 1991). PCR was conducted for 10 µl of 10X PCR reaction buffer (Fermentas, Germany), 200 µM of each dNTP, 1 µM of each primer and 5 units of AmpliTaq DNA polymerase in a final volume of 100 µl. Amplification was done in a Bio-Rad thermal cycler under the following conditions: 5 min of initial denaturation at 94°C, followed by 35 cycles of amplification with a 1 min denaturation at 94°C, 1 min annealing at 58°C, 1.5 min extension at 72°C and a final extension step of 7 min at 72°C (Suzuki and Yamasato, 1994). PCR products were analyzed by 1% agarose gel electrophoresis. After staining with gel red, the results were observed by gel-document (Syngene, USA).

Sequencing and phylogenetic analysis. The amplified DNA fragments were purified by gel electrophoresis in 1% agarose, recovered with a high pure PCR product purification kit (Roche, Germany) and custom sequenced (MWG, Germany). The 16S rRNA sequences were compared with available 16S rRNA sequences in Gene Bank using the BLAST search facility at NCBI. *B. subtilis* strain and reference sequences were aligned with ClustalW (Thompson *et al*., 1994) and alignments were subsequently adjusted manually in BioEdit v.7.0.9 (Hall, 1999). Phylogenetic relations were inferred applying the Kimura-2-parameter model (Kimura, 1980) with the neighbor joining (NJ) algorithm (Saitou and Nei, 1987) as implemented in MEGA4 (Tamura *et al*., 2007). The confidence of branching was assessed by computing 1000 bootstrap (Felsenstein, 1985).

Phenotypic and biochemical experiments. Strain identification was based on traditional phenotypic methods including Gram staining, motility, endospore and sporangia production, spore position, oxidase reaction, catalase activity, starch hydrolysis, presence of lecithinase, casein hydrolysis, nitrate reduction, anaerobic growth, gelatin hydrolysis, citrate utilization, acid production from D-glucose, L-arabinose, D-manitol and D-xylose, growth at 4 and 45ºC, methyl red-voges proskaure, growth at pH 5.7 and 6.8, growth in 5% and 7% NaCl (Bergey *et al*., 1986).

Antifungal activity of the bacterial strains. Antifungal activity of the studied bacteria was evaluated using the spot inoculation method (Kumar *et al.*, 2009) with minor changes. Briefly, bacteria were spotted on the PDA medium and allowed to grow at 37ºC for 48 h. Then, a 5 mm plug was removed from the margin of a 5-day fungal culture, placed in the center of plate and incubated at 27ºC for 2 days. Control plates were cultured only with a *F. culmurum* plug. Each experiment was run in triplicate and repeated at least three times. Results were measured as mean % inhibition ± standard deviation of the *F. culmurum* growth in the presence of each bacterial strain. Antifungal activity was measured according to the difference between the diameter of fungal colonies in the presence or absence (control) of the bacterium.

Percentage of fungal growth inhibition was calculated according to the following formula:

$$A\text{-}B/A\times100$$

Where A = diameter of the fungal colony in the control; B = diameter of the fungal colony in the presence of bacteria. Data were analyzed using ANOVA and the Duncan Multiple test ($P<0.05$).

Detection and antifungal activity assay of volatiles. A Petri dish containing NA and 2% glucose (NAG) was inoculated by spreading 200 µl of suspension of each bacterial strain, prepared from 72-h NA culture and incubated at 37ºC for 24 h. A 5 mm plug of *F. culmorum* colony was cultured in the center of a PDA plate, then inverted and placed over the bacterial plate in sterile condition. The two plates were sealed together with parafilm and further incubated at 28ºC for 5 days. Finally the diameters of fungal colonies were compared with those of controls. In the control plate, sterile water was used instead of bacterial suspension. Antifungal activity was measured as mentioned above (Fiddaman and Rossall, 1993).

Determination of protease activity. The bacterial strains were grown in 50 ml NA (24 h, at 37ºC on shaker) then centrifuged at 13,870 *g* for 5 min at 4ºC. For each strain 5 µl of supernatant was spotted on skim milk agar (1% skim milk, 0.02% sodium azide and 2.0% agar). Protease activity of the strains was detected by observing and measuring of clear zones around the culture after 24 h (Chantawannakula *et al*., 2002).

Microtiter plate assay of pellicle formation. All bacterial strains were grown in LB medium overnight at 37ºC on shaker, then diluted in the same fresh medium. After reaching OD_{575} = 1, the cultures were diluted 350-fold in minimal MSgg medium [5 mM potassium phosphate, 100 mM MOPS (morpholine propane sulfonic acid pH 7), 2 mM $MgCl_2$, 700 µM $CaCl_2$, 50 µM $MnCl_2$, 50 µM

$FeCl_3$, 1 µM $ZnSO_4$, 2µ M thiamine, 0.5% glycerol, 0.5% glutamate, 50 µg ml^{-1} tryptophan, 50 µg ml^{-1}, phenylalanine, and 50 µg ml^{-1} threonine] supplemented with 200 µM NaCl. Bacterial cells were incubated in 96-well polystyrene plates at 37ºC for 70 h without shaking. The medium was removed and wells were rinsed with water and allowed to dry at 37ºC for 15 min. Methanol was then added to fix the biofilm to the walls of the wells. After methanol removal and rinsing the wells with water and drying, 1% crystal violet (CV) solution was added for staining bacterial cells adhering to the wells. After 5 min, excess CV was removed and the wells were rinsed with water. In the final step an ethanol-acetone solution (4:1, vol/vol) was added. Biofilm formation was quantified by measuring the OD_{500} for each well using a plate reader (Nagórska *et al*., 2008). The medium without bacteria was used as a negative control. Each treatment was repeated 3 times.

Preparation of seeds treated by antagonistic bacteria. Selected bacteria were cultivated overnight in LB medium, then centrifuged 4,990 *g* for 10 min. Wheat seeds (*Triticum aestivum*) cv. Falaat were shaken for 2 h in a suspension of antagonistic bacteria (1×10^9 CFU) with 1% methylcellulose, then dried on filter paper under sterile air flow for 1 h. Seeds inoculated with antagonistic strains were used for biocontrol assays in greenhouse pot tests (Kempf and Wolf, 1989).

Biocontrol of *F. culmorum* in greenhouse. Inoculum was prepared by incubating 30 g of wheat seeds in 400 ml tap water with 10^7 macroconidia of *F. culmorum* for 7 days at 27ºC on a rotary shaker. Sterile sand-peat-soil (1,300 g) was blended with 3 g of seed colonized with fungal inoculum and placed in plastic pots (6×6×7 cm). Seeds treated with antagonistic bacteria were sown in soil inoculated with the fungal pathogen (Kempf and Wolf, 1989). Wheat seeds without bacteria and fungus were used as healthy control whereas seeds exposed to the fungus represented the infected control. Water was added every 2-3 days. Each treatment was replicated three times and the experiment was conducted twice. Disease severity was evaluated after 4 weeks. The disease index was calculated according to the following arbitrary scale: 0 = healthy plants; 1 = slightly brown coleoptiles/roots; 2 = moderately brown coleoptiles and roots; 3 = severe browning of coleoptile and roots; 4 = dead plants. The effects of antagonistic bacteria on the foliage and root dry weigh were taken into account in scoring biocontrol effects (Knudsen *et al*., 1995).

RESULTS

With specific PCR amplification of the 16S rRNA gene, the expected 1.5 kb band was amplified from all bacterial strains. The partial sequences (600-900 bp) obtained were deposited in GenBank under accession numbers HQ197378, HQ234318-32 and HQ267751-64 (Table 1). Sequences of all strains had more than 98% identity with those of *Bacillus* sp. strains from Gene Bank. Phylogenetic analysis of the 600 bp 16S rRNA region allowed distinguishing different groups within the tested strains. The phylogentic tree comprised two *B. subtilis* reference sequences (FJ435215 and AB526464) and *B. licheniformis* sequence (EU 697936) used as outgroup. All strains clustered in one large group that was split into subgroups A and B (Fig. 1).

Results of biochemical and phenotypic tests showed all the strains were Gram-positive, produced endospore located in the center of the body but not swell sporangia, were mobile and grew at 45ºC but not at 4ºC. Oxidase reaction, starch hydrolysis, citrate utilization, lecithinase,

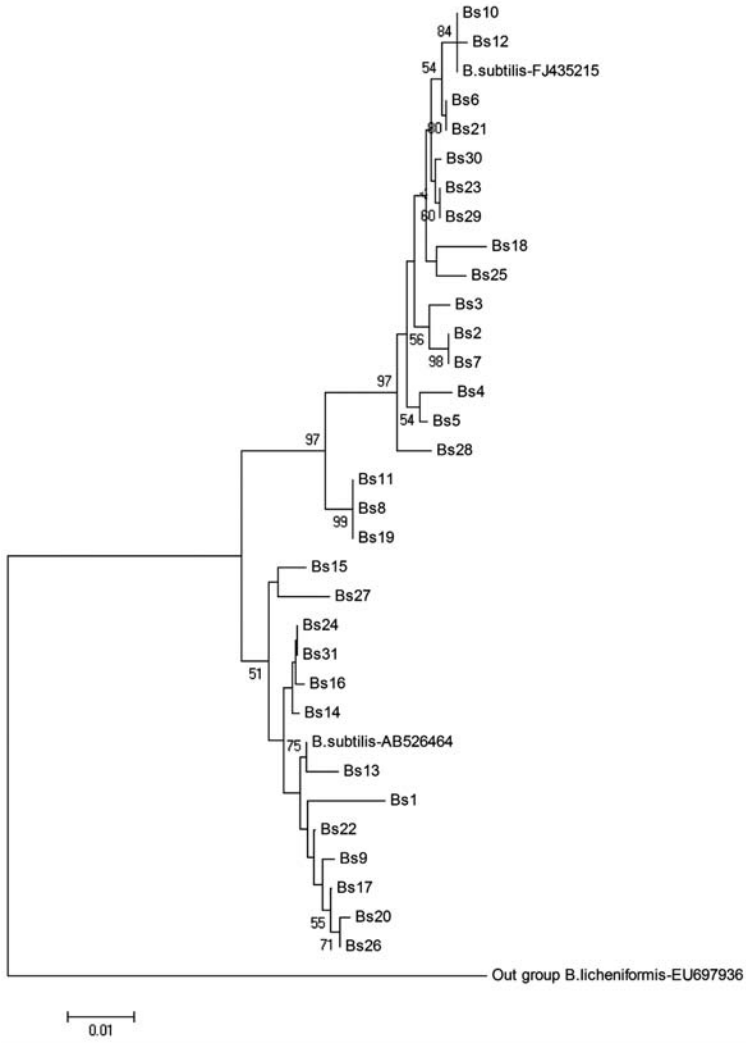

Fig. 1. Phylogenetic relationship of *B. subtilis* strains. Bootstrap values (expressed as percentages of 100 replications) are shown at branch points. The bar represents the unit length of the number of nucleotide substitutions per sites. Abbreviations, B: *Bacillus* and Bs: *Bacillus subtilis.*

Table 2. Phenotypic tests of the strains and comparison with closely related *Bacillus* species.

Bacteria	Studied strains	*B. subtilis* (DSM No. 3258)	*B. cereus**	*B. thuringiensis**	*B. megaterium**
Characteristic					
Gram reaction	G^+	G^+	G^+	G^+	G^+
Motility	+	+	+	+	+
Spore position	central	central	subterminal/ paracentral	subterminal/ paracentral	central/paracentral/ subterminal
Swell sporangia	-	-	-	-	-
Oxidase reaction	+	+	-	-	nd***
Catalase activity	+	+	+	+	+
Hydrolysis of:					
Casein	+	+	+	+	+
Gelatin	+	+	+	+	+
Starch	+	+	+	+	+
Nitrate reduction	+	+	d**	+	d
citrate utilization	+	+	+	+	+
Anaerobic growth	-	-	+	+	-
Growth at:					
4°C	-	-	-	-	d
45°C	+	+	+	+	d
pH= 5.7	+	+	+	+	+
pH= 6.8	+	+	+	+	+
NaCl 5%	+	+	+	+	+
NaCl 7%	+	+	d	+	+
Voges proskaure	+	+	+	+	-
Lecithinase	-	-	+	+	-
Acid production from:					
D-glucose	+	+	+	+	+
L-arabinose	+	+	-	-	+
D-manitol	+	+	-	-	+
D-xylose	+	+	-	-	+

* Reactions are based on Bergey *et al*. (1986); ** d, different strains give different reaction; *** nd, no data are available.

gelatin hydrolysis, nitrate reduction, catalase activity, casein hydrolysis and voges proskaure test were positive, whereas anaerobic growth was negative. All strains grew in 5% and 7% NaCl and at pH 5.7 and 6.8 and produced acid from D-glucose, L-arabinose, D-mannitol and D-xylose. Based on these results and following comparison with closely related species in genus *Bacillus* all the Iranian strains were identified as *B. subtilis* (Table 2).

Results of laboratory bioassays for assessing the antagonistic potential of rhizobacterial strains showed that 86% of them had more than 64% inhibitory effect on mycelial growth of *F. culmorum*. Additionally, they possessed different protease activity as indicated by the clear zone around the colonies that ranged between 3.66 and 9.66 mm. Strains Bs9 (9.66 mm), Bs10 (8.66 mm) and Bs7 (6 mm) showed the maximum protease activity on skim milk, whereas the minimum protease activity was shown by Bs11 and Bs18 with a 3.66 mm clear zone.

Strain Bs20 produced the maximum and strains Bs5 and Bs31 the minimum amount of volatile compounds. It is important to note that 46% of the strains producing high volatile quantities, showed more than 50% inhibitory effect on *F. culmorum* (Table 3).

Evaluation of biofilm formation showed that all the studied strains produced pellicle but the amount of biofilm was remarkably different. The highest biofilm production was by strains Bs1, Bs4 and Bs12 and the lowest by strains Bs31, Bs7 and Bs25.

Fifteen strains were selected to evaluate the correlation among antagonistic laboratory experiments, biofilm formation and antagonistic power against *F. culmoroum* in greenhouse experiments. Among these, there were strains with low, moderate and high antagonistic power as well as biofilm production in laboratory (Table 3).

Root rot symptoms were observed 4 weeks after the inoculation of wheat. The records included disease severity and dry weight of foliage and root in different treatments. All the studied bacteria affected the disease, their biocontrol effect being significantly different compared with the control at 1% probability. Strain Bs1 showed the most antagonistic effect (100%) on common root rot so that no disease symptoms were observed. Plants exposed to strains Bs4 and Bs5 (89% biocontrol activity) looked almost like healthy controls, a condition comparable to that consequent to inoculation with strains Bs11 and Bs12 (81% biocontrol activity). The least inhibitory effect (33%) was afforded by the strains Bs9 and Bs31.

Comparison of the strains in the phylogenetic dendrogram showed that there was not any correlation among the studied characteristics of the strains that clustered in two subgroups.

Based on our results, four *B. subtilis* strains (Bs1, Bs4, Bs5 and Bs12) that show profuse biofilm production and antagonistic effects on common root rot in greenhouse are eligible for selection and use for more detailed studies and field trials for their practical utilization as potential biocontrol agents.

DISCUSSION

Numerous studies have documented the deleterious effects and environmental risks of fungicides used for biocontrol of FHB in the field (Müllenborn *et al*., 2008; Menniti *et al*., 2003; Mesterhazy *et al*., 2003; Khan *et al*., 2004). Therefore, application of fungicides is not recommended for controlling common root rot, except in areas with an overall high risk of infection. Thus, using effective biological control agents is the best way to decrease damage by *F. culmorum* and other FHB agents in wheat crops. Different members of the genus *Bacillus* have received increasing attention because of their beneficial effects on plant growth consequent to nutrient mobilization and biological control of different diseases and pests in the field.

B. subtilis is well known for its antifungal properties and biological control of a number of plant and animal diseases (Chaurasiaa *et al*., 2005). So, in the present study we attempted to select and characterize some Iranian *B. subtilis* strains as BCAs against wheat common root rot, an important disease in the country (Mohammadi *et al*., 2005) and to find correlations between some biochemical and physiological characteristics of the isolates, such as biofilm formation, volatile production and protease activity, and their antagonistic power in laboratory and greenhouse. The studied strains showed different antagonistic effects on mycelial growth of *F. culmorum* in dual culture and greenhouse experiments. In addition, some of the most powerful antagonistic strains like Bs1 produced a large quantity of secondary metabolites and biofilm (Table 3).

Production of secondary metabolites, especially bacilycin, iturin A, fengymycin, bacillumycin and mycobacillin by *Bacillus* spp. strains has been reported as one of the most important mechanisms inhibiting

Table 3. Effect of selected antagonistic *B. subtilis* in laboratory and greenhouse experiments.

Strains	Antagonistic power (%)*	Volatile (%)*	Protease (mm)	Biofilm (OD500)	Biocontrol greenhouse (%) ***
Bs1	64.87^{D}**	84.48^{A}	4.33^{FGH}	1.604^{A}	100
Bs3	18.64^{E}	86.28^{A}	4.66^{EFG}	0.956^{ABC}	62
Bs4	$73.11B^{CD}$	42.65^{C}	4^{GH}	1.584^{A}	89
Bs5	$78.1B^{C}$	0^{G}	3.66^{H}	1.371^{AB}	89
Bs7	91.21^{A}	37.52^{C}	6^{C}	0.11^{D}	61
Bs9	81.42^{B}	55.54^{B}	9.66^{A}	0.088^{D}	33
Bs10	89.77^{A}	16.53^{DE}	8.66^{B}	0.132^{D}	52
Bs11	14.3^{EF}	54.2^{B}	3.66^{H}	0.453^{DC}	81
Bs12	79.34^{BC}	23.1^{D}	5.66^{CD}	1.477^{A}	83
Bs15	70.62^{CD}	11.9^{EF}	4.66^{EFG}	0.751^{BCD}	37
Bs18	81.42^{B}	4.8^{FG}	3.66^{H}	0.236^{D}	41
Bs20	70.62^{CD}	88.21^{A}	5.33^{CDE}	0.754^{BCD}	67
Bs25	79.34^{BC}	54.2^{B}	5^{DEF}	0.038^{D}	39
Bs26	70.62^{CD}	85.53^{A}	5.66^{CD}	0.111^{D}	48
Bs31	75.58^{BC}	0^{G}	4.66^{EFG}	0.083^{D}	33

* Percentage of mycelia growth reduction measured after complete growth of the *F. culmorum* in control plate.

** Numbers with similar letter in each column are not statistically different at 1% level according to Duncan's range test (numbers are average of three replications).

*** Percentage of biocontrol comparing healthy control (treatment without fungus and bacterium).

growth of soil-borne plant pathogens, especially FHB agents (Johansson *et al*., 2003; Schreiber *et al.*, 1988; Wang *et al*., 1992). All the antagonistic strains we studied were able to produce volatile compounds. Fiddaman and Rossall (1993, 1994) had shown that production of volatile compounds by *B. subtilis* could decrease the mycelial growth of *Pythium ultimum* and *Rhizoctonia solani* and that volatile production and antagonistic power of the bacterium increases in media containing a large amount of sugars. We have now shown that volatile compounds produced by 46% of the tested *Bacillus* strains grown on NAG medium induced over 54% inhibition of fungus mycelia.

Protease production by BCAs such as *P. fluorescens*, *Stenotrophomonas maltophilia* and *T. harzianum* is another of the important factors of antagonistic activity against plant pathogenic bacteria and fungi (Dunne *et al*., 2000; Elad, 2000; Howell, 2003; Kobayashi *et al*., 2005; Siddiqui *et al*., 2005). All strains tested had a protease activity as shown by the induction in the cultures of a clear zone with radius more than 4 mm in size within 24 h. According to Cooper (1963), activity of biological substances (i.e. antibiotics and enzymes) can be expressed in terms of the square of the diameter of the clear zone.

However, we found strains with high protease activity that did not decrease disease symptoms in the greenhouse, maybe because of their different adaptation to greenhouse environment (Table 3). In fact, some rhizosphere bacterial strains that have antagonistic effects on plant pathogenic fungi when tested in laboratory cultures on synthetic media plates can also show good disease suppression under glasshouse conditions depending on factors related to strains and environmental conditions (Johansson *et al*., 2003).

Plant roots and rhizosphere are preferential sites for colonization by soil microorganisms, since root exudates and death or lysis of cortex cells during root growth supply large amounts of nutrients, i.e. sugars, amino acids and organic acids, that can serve as energy, carbon and/or nitrogen sources (Molina *et al*., 2003). Different kinds of bacteria can grow and produce in these ecological niches biofilms that facilitate survival and adaptation to variable environmental conditions. There are not many documented studies on the direct effect of biofilm formation on biological control of rhizobacteria, but previous studies have reported that biocontrol mechanisms could be related to biofilm formation in the rhizosphere (Bais *et al.* 2004; Morikawa, 2006; O'Toole and Kolter, 1998). Results of our greenhouse studies showed that strains with high biofilm productivity, (Bs1, Bs4, Bs5, Bs12) had the highest antagonistic effects, whereas the contrary was true for strains with low biofilm productivity (Bs9, Bs15, Bs18, Bs25, Bs26, Bs 31). The high antagonistic activity of biofilm-forming strains may be linked with their successful establishment and persistence on the roots as a result of biofilm formation. A previous study by Bais *et al.* (2004) showed that ability of biofilm formation in *B. subtilis* has direct effect on antagonistic power against plant bacterial pathogens. They reported that *B. subtilis* 6051 protected *Arabidopsis* against *P. syringae* pv. *tomato* under *in vitro* and soil conditions. This antagonistic isolate could produce surfactin and biofilm, whereas its surfactin-deficient mutant was inactive in biofilm formation and biocontrol, because secretion of surfactin is in connection with biofilm formation. Surfactin has antimicrobial activity and at high concentration kills the pathogens. The effect of surfactin on *B. subtilis* biofilm formation was positive whereas the contrary is true for *Salmonella enterica*, *Escherichia coli* and *Proteus mirabilis*. According to Bais *et al.* (2004), it is possible that the presence of *B. subtilis* 6051 surfactin may prevent the planktonic cells of other microbes like *P. syringae* pv. *tomato* from colonizing *Arabidopsis* roots. As shown here, correlation between biocontrol of disease in the greenhouse and biofilm formation was 53% and significant difference of 1% among strains was observed. Several authors have shown that effectiveness of biocontrol microorganisms is directly dependent on their efficiency in root colonization (Molina *et al*., 2003; Thomashow, 1996). In addition, colonization has been claimed to be related to biofilm formation and this phenomenon constitutes a strategy for bacteria to survive desiccation or other environmental stresses and actively participates in defense mechanisms involved in pathogenic attacks by other microorganisms (Yaryura *et al*., 2008; Timmusk *et al*., 2005). In the phylogenetic tree, all strains were clearly separated from the outgroup strain, and were divided into two subgroups. We could not find any common characteristics between the strains in each subgroup with the results of laboratory or greenhouse experiments.

Finally, based on our study, it is clear that the antagonistic activity of BCAs is the result of complex mechanisms which interact with each other and it is not possible to relate antagonism efficiency with any one of these mechanisms singly. Selection of effective BCAs based on their different biochemical and physiological characteristics is an important step in the biological control of plant diseases. As mentioned, four of our *B. subtilis* strains, i.e. Bs1, Bs4, Bs5 and Bs12 showed a high correlation between biofilm formation and control of common root rot of wheat in the greenhouse. Thus, the ability of biofilm formation can be considered as one of the important factors to be taken into consideration for selection of bacterial BCAs for practical utilization.

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