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Bacillus subtilis Strains with Antifungal Activity against the Phytopathogenic Fungi

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Abstract

Bacillus strains isolated from the rhizosphere soil of potato roots were evaluated for the potential antagonistic activity against fungal pathogens *in vitro* and *in vivo*. Two bacterial isolates were identified as new *Bacillus subtilis* strains by 16S rRNA and GyrB gene sequencing and were designated GM2 and GM5, respectively. Strains were characterized by their ability to inhibit growth of a number of phytopathogenic fungi. It was shown that GM5 strain inhibited growth of phytopathogenic fungi more effectively than GM2 strain. Both strains were capable of producing a number of hydrolytic enzymes as well as antimicrobial metabolites (ammonia and HCN). In addition, GM2 strain also produced siderophores. Four genes encoding antimicrobial peptides were identified in the genome of GM2 strain: *ituC*, *bmyB*, *bacA* and *srfA*. Genome of GM5 contained two genes encoding for antimicrobial peptides, *srfA* and *fenD*. Purified lipopeptide fraction from GM5 but not from GM2 strain was able to control *Fusarium solani* spread in the plate assay. Furthermore, *Bacillus subtilis* strain GM2 promoted growth of wheat but only GM5 strain was able to protect wheat seedlings from *Fusarium oxysporum* infection.

Keywords

Bacillus subtilis, *Fusarium*, Phytopathogenic Fungi, Antagonistic Activity, Antimicrobial Peptides

1. Introduction

Biological control is an environmentally-friendly alternative to chemical pesticides and it is an attractive method protecting the plants from pathogens, because the wide usage of chemicals has a negative impact on the environment and

human health. Many biocontrol agents were isolated by screening of the large number of soil or plant-associated microorganisms for antagonism against phytopathogens *in vitro* or *in planta* [1] [2]. A number of bacterial species with antagonistic activity were isolated from the rhizosphere of different plants. Among those, bacilli and pseudomonads are the most common isolates [1] [2] [3] [4].

It is known that various species of the *Bacillus* genus are able to stimulate the plant growth [5]. Bacteria can promote plant growth through a number of mechanisms, such as improvement of plant nutrition; induction of systemic resistance; toxicity to pests and antagonism pathogens [5] [6] [7]. The antagonistic activity of *Bacillus* is associated with the synthesis of various antimicrobial peptides [8] [9], secreted enzymes [10], proteins [11] and volatile organic compounds (VOCs) [5] [10]. Many *Bacillus* isolates were shown to have antifungal activity against phytopathogenic fungi that make them good biocontrol candidates [12] [13] [14]. Cyclic lipopeptide fengycin plays an important role in this process [15].

The strains of the species *B. subtilis* can vary considerably both phenotypically and genetically and that affects their antagonistic potential. Comparative analysis of proteomes of two *B. subtilis* strains with antagonistic potential highlighted the major differences in the composition of intracellular and extracellular proteins [11] [16], some of which can be associated with antimicrobial properties. Because of their fast growth, ability to effectively grow in low cost media and to sporulate under undesirable conditions *Bacillus* isolates are the attractive candidates for application as the biocontrol agents. There is a growing demand for such agents since it is expected that global market for biopesticides will significantly expand in the next 3 - 5 years (www.bccresearch.com/market-research/chemicals/biopesticides-chm029e.html; [17]).

The aim of this study was to directly compare two antagonistic strains of *Bacillus* spp. isolated from potato roots, in their ability to suppress phytopathogenic fungi through production of cyclic lipopeptides, hydrolytic enzymes and siderophores. In addition, we evaluated plant growth promoting potential of *B. subtilis* GM2 and GM5 on wheat seedlings. Finally, we showed that one of the two *B. subtilis* isolates, GM5, can protect wheat seedlings against *Fusarium oxysporum* and, therefore, represents a biocontrol candidate with the potential for its application in agriculture.

2. Material and Methods

2.1. Isolation and Identification of Antagonistic Strains

Several bacterial strains were isolated from the rhizosphere of potato roots. The non-rhizosphere soil was removed by gentle shaking. The rhizosphere-associated soil was collected from roots by dipping and gentle shaking in sterile water under aseptic conditions. The resulting soil suspension was used to inoculate LB agar plates and pure cultures were obtained by repetitive streaking to single colonies on the additional LB plates. Plates were incubated for 48 h at 28˚C. For

long-term storage bacterial strains were kept at −70˚C in LB-broth with 15% glycerol (v/v).

The colonies with different colony morphologies were selected for further studies. Bacterial isolates were screened for their activity against *Fusarium* sp. as described in section *In vitro* antagonistic activity. Two bacterial isolates with the highest inhibitory activity were selected for further characterization.

Pure bacterial cultures grown overnight at 30˚C were analyzed by MALDI BioTyper (Bruker Daltonik). This technique is based on the comparison of a number of bacterial proteins against preexisting database. Scores were calculated by Biotyper software as arbitrary units with values between 0 and 3 as a result of comparison of each sample mass spectrum to the reference mass spectra in the Bruker database. Species identification is possible when the of score values lie between 2.300 and 3.000 according to manufacturer's recommendations. Genomic DNA was isolated by standard technique [18]. The 16S rRNA genes were amplified by PCR using primers: Fw 5'-gagtttgatcctggctcag, Rev 5'-acggttaccttgttacgactt. DNA gyrase subunit B genes were amplified with the following primers: UP1 5'-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA and UP2r 5'-AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT. PCR analysis was performed according to the established protocol [19]. PCR products were separated on agarose gel [18].

The obtained sequences were analyzed using the BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2. Phytopathogenic Fungi and Culture Condition

In this study we used the following phytopathogenic fungi: *Fusarium avenaceum*, *Fusarium oxysporum*, *Fusarium redolens*, *Fusarium solani*, *Fusarium* sp., *Alternaria tenuissima* (all from Kazan Federal University Department of Microbiology fungal collection, Kazan, Russia), *Alternaria alternata* TP 712, *Alternaria solani* 12RKL15, *Doratomyces* sp. 14raKKLD and *Colletotrichum coccodes* 14raKK6 (all from Lomonosov Moscow State University Department of Microbiology fungal collection). The fungi were cultivated in the Czapek medium ($NaNO_3$, 3.0 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; agar, 15 g; distilled water, 900 ml; pH 4.5) [20]. The plates were incubated at 28˚C for 5 - 14 days.

2.3. *In Vitro* Antagonistic Activity Assay

The interaction studies of rhizobacteria and phytopathogenic fungi were performed using the *in vitro* dual-culture analysis. Mycelial discs with 8 mm diameter were cut from the target fungi colonies cultured on Czapek plates for seven days and were placed on fresh Czapek plate. Lawns of bacterial individual strains were grown on LB plates for 48 h and were excised from the plate with a sterile scalpel (8 mm). Bacterial blocks were placed at the distance of 3 cm from the fungal discs on the same agar plate. Control plates without bacterial strains were prepared simultaneously. Plates were incubated at 28˚C for 7 - 14 days and

examined for the inhibition of fungal growth. To calculate the percent of inhibition we repeated these experiments three times. The growth inhibition of the test fungus was calculated using the following formula:

$$\text{Inhibition}(\%) = \left[(R - r)/R \times 100\right] \quad (1)$$

where

R—(a control value) represents the radial growth of fungus in control sets.

r—the radial growth of the fungus in sets with bacteria.

2.4. Antagonistic Activity of Extracellular Bacterial Metabolites

Czapek broth was used in studies of antagonistic activity of extracellular bacterial metabolites against the micromycetes. Bacterial cultures were grown in LB broth at 37˚C for 3 days. Cells were removed by centrifugation at 13,000 *g* for 30 min and then supernatant was filtered through sterile 0.22 microns membrane filter (CAMEO®, GVS, Italy). The resulting supernatant was added to the Czapek broth at the 1 to 4 ratio (10 ml of bacterial culture and 40 ml of Czapek medium). The fungal spores were added at the concentration of 2×10^7 and cultivated aerobically at 28˚C for 6 days on the shaker at 60 rpm (IKA®KS 4000, Germany). Next, fungal cultures were filtered through a filter paper (Whatman No. 1), dried and weighed. Czapek broth without bacterial metabolites and treated in the similar fashion to as described above served as a control. To study temperature stability of metabolites with inhibitory activity bacterial cell-free culture filtrate collected as described above was subjected to autoclave sterilization (30 minutes at 121˚C). The percent of the weight reduction of test fungus was calculated using the following formula:

$$\text{Inhibition}(\%) = (W_1 - W_2)/W_1 \times 100 \quad (2)$$

where

W_1—the weight of the test fungus in control flasks (without bacterial metabolites).

W_2—the weight of the fungus in flasks with media containing bacterial metabolites.

2.5. Microscopy of Fungal Mycelia

The morphological changes caused by antagonistic bacteria on the mycelia of the phytopathogenic fungal species (*Fusarium* sp., *A. alternata*) after culturing on Czapek plates for 6 - 7 days were directly examined under optical microscope (XSZ-148E, Russia, under 40× magnification).

2.6. Production of Hydrolytic Enzymes and Antifungal Metabolites

Proteolytic activity was determined in well-established plate assay [21] based on the appearance of zones of clearance in the LB-agar containing skim milk (10%). The amylolytic and pectinolytic activities were evaluated on plates containing minimal M9 media [18] supplemented with 0.5% yeast extract and 0.2% soluble

starch (v/w) or 0.5% pectin (v/w) to test for amylase and pectinase, respectively. Plates were incubated at 30˚C and after 2 - 3 days were overlaid with 0.1% Lugol's iodine to identify clear zone formation.

Cellulase activity was assayed on indicator plates containing 0.5% carboxymetylcellulose (CMC) [22]. Plates were incubated at 28˚C for 72 h and then were extensively washed to remove bacteria off the media surface. Next, plates were stained with 1 $g \cdot l^{-1}$ Congo Red solution for 30 min. Formation of clear zones around the colonies indicated CMC degradation.

Siderophore production was assessed on chrome azurol S (CAS) agar plates by observing the color change from blue to orange. The plates were incubated at 30˚C for 5 - 7 days [22].

Ammonia production was tested in colorimetric assay. Addition of 1 ml of Nessler's reagent to the 72-h-old bacterial culture grown in peptone broth resulted in the development of the yellowish-brown color if ammonia are present in the media [23].

HCN production of the isolates was detected by the method of Bakker and Schippers [24]. Briefly, HCN production was determined by the color change of the filter paper previously dipped in 2% sodium carbonate/0.05% picric acid solution and placed on the bacterial lawn. Brown color indicated the presence of HCN.

2.7. PCR Amplification of the Genes Responsible for the Synthesis of Antimicrobial Peptides

The genes responsible for the synthesis of antimicrobial metabolites were identified by PCR amplification using specific primers to sequences of genes *srfA* (surfactin), *bacA* (bacilysin), *fenD* (fengycin), *bmyB* (bacillomycin), *ituC* (iturin). Partial sequence of *ituC* (acyl-protein synthetase ItuC) gene from *B. subtilis* B010 (http://www.ncbi.nlm.nih.gov/nuccore/262182344) with the size of 468 b.p. was used to design primers for *ituC* gene; *B. subtilis* QST713 (353 b.p., http://www.ncbi.nlm.nih.gov/nuccore/DQ011330.1) sequence was used for *bmy*B (peptide synthase BmyB) gene; *B. subtilis subsp. spizizenii TU-B-10* (615 b.p., http://www.ncbi.nlm.nih.gov/gene/11241385) sequence was used for *bac*A (bacilysin biosynthesis protein BacA) gene; *B. subtilis subsp. subtilis* 168 (10764 b.p., http://www.ncbi.nlm.nih.gov/gene/938306) sequence was used for *srf*A (surfactin synthase subunit 1 SrfA) gene; *B. subtilis* (7774 b.p., http://www.ncbi.nlm.nih.gov/nuccore/AJ011849.1) sequence was used for *fen*D (fengycin synthetase FenD) gene.

Multiple alignments of the genes involved into antimicrobial peptide biosynthesis for sequenced *B. subtilis* strains present on the NCBI server (http://www.ncbi.nlm.nih.gov/) were performed to design primers to the conservative regions (**Table 1**).

The annealing temperature of primers was calculated using the program OligoCalc (http://www.bio.bsu.by/molbiol/oligocalc.html). Amplification of these marker genes showed that each gene had one specific band of the expected size.

Table 1. Primers for antimicrobial peptide biosynthesis genes.

Name	Sequence	Annealing temperature, ˚C	Expected size, bp	Gene product
ituC-F	TGCCATTATTGTCTACGGAG	50	270	acyl-protein synthetase ItuC
ituC-R	ATAAATCATACAGCCGAC	43		
bmyB-F	ACGGCAGGTTTTGATTTTT	45	290	bacillomycin L synthetase BmyB
bmyB-R	CGTTCCTTATCTCCGGA	47		
bacA-F	CATTTCCAATTTTACTCTTC	44	410	bacilysin biosynthesis protein BacA
bacA-R	TACTTTTGCCGTGCAAGCTC	52		
srfA-F	AACGGGGAGCCTGTTCAATA	52	420	surfactin synthase subunit 1 SrfAA
srfA-R	ACAAGTTCAGGCACCGATTC	52		
fenD-F	AAAGGTGTGTGGAATTGATG	48	430	fengycin synthetase FenD
fenD-R	GCTGTCTCCTCTATCAAAAA	48		

2.8. Lipopeptide Fraction Preparation

B. subtilis strains GM2 and GM5 were grown in 200 ml of LB-broth at 30˚C for 72 h. Bacteria were removed by centrifugation at 12000 g for 30 min at 4˚C followed by filtration of conditioned media through 0.22 μm filters (Millipore). pH was adjusted to 2.5 using 6N HCl. Acidified conditioned media were subjected to centrifugation at 10,000 g for 30 min at 4˚C. Resulting pellets were dissolved in methanol-water (50/50), incubated for 3 h at room temperature and spun down by centrifugation at 10,000 g for 10 min. Methanol-soluble fraction was transferred to a new tube and dried under vacuum at 45˚C for 3 h. Dried samples were dissolved in 50 μl of methanol-water (50/50) and used for HPLC analysis. An equal volume of LB-broth was treated in a similar fashion and used as a negative control.

2.9. HPLC Analysis

Lipopeptides were dissolved in methanol and separated by HPLC using Acclaim® Polar Advantage II (PA2) C18 reverse-phase column (5 μm, 250 × 4.6 mm) and UltiMate 3000 UHPLC system (Thermo Scientific, Dionex, USA) as described by Roy *et al.* [25].

2.10. Antifungal Activity of Lipopeptide Fraction

Antifungal activity of HPLC-purified lipopeptide fraction from *B. subtilis* GM2 and GM5 was determined by disc diffusion assay. Mycelial discs with 8 mm diameter were cut from the target fungi colonies of *F. oxysporum* and *F. solani* cultured on Czapek plates for seven days and were placed on fresh Czapek plate. 10 μl of each lipopeptide fraction (see sections Lipopeptide fraction preparation and HPLC analysis) were placed on the sterile filter discs (6.5 mm). Methanol-PBS (50/50) was used as a control. Filter discs were placed 3 cm away from the fungus and plates were incubated at 30˚C for 6 days. Formation of growth inhibition zone around lipopeptide-containing discs indicated the presence of anti-

fungal activity.

2.11. Plant Growth Promotion and Antifungal Activity of *B. subtilis* GM2 and GM5 on Wheat Seedlings

Wheat seeds were sterilized in 70% ethanol for 30 s, washed three times with sterile water, incubated for 5 min in 2.5% sodium hypochlorite, washed again with sterile water three more times and air dried. Sterilized seeds were placed on sterile filter paper soaked in sterile water and incubated in a Petri dish at room temperature for 24 h. Twenty seedlings were selected for each experimental group. To evaluate plant growth promotion by *B. subtilis* GM2 and GM5 seedlings were incubated either with water or with 3-days old bacterial suspension (10^7 CFU/ml) for 1 h, placed on water soaked filter and incubated in sterile Petri dish at 25˚C for 8 days.

To evaluate antifungal activity of *B. subtilis* GM2 and GM5 8 mm mycelial discs of 7-days-old *F. oxysporum* (see section *In vitro* antagonistic activity assay) were placed in 500 ml of sterile water and incubated for 30 minutes at room temperature with shaking (200 rpm) to release spores. Resulting spore suspension was further diluted to 10^5 CFU/ml. Wheat seedings were placed on the sterile paper filters soaked either in water or in 5 ml of spore suspension and incubated in the Petri dishes for 8 days at room temperature. Seedling survival was expressed in percent of total number of seedlings in each group. Root and shoot dry weights were measured and the average values were calculated and compared to corresponding weights obtained for seedlings from the control group.

3. Results

We isolated 48 bacterial isolates from three independent potato plants rhizosphere characterized by different colony morphology. Twenty five isolates were identified as Gram positive bacteria; the remaining 23 isolates had Gram negative staining. Organism identification by MALDI BioTyper showed that *Bacillus* species were predominant among Gram positive bacteria: *Bacillus subtilis* (14 isolates), *Bacillus pumilus*, *Bacillus weihenstephanensis*, *Bacillus thuringiensis.* Other Gram positive bacteria included *Lysinibacillus fusiformes*, *Lysinibacillus sphaericus*, *Brevibacterium iodinum.* Among Gram negative bacteria were identified *Acinetobacter calcoaceticus*, *Citrobacter freundii*, *Enterobacter amnigenus*, *Enterobacter cloacae*, *Enterobacter ludwigii*, *Myroides odoratus*, *Providencia alcalifaciens*, *Serratia rubidaea*, *Serratia plymuthica*, *Serratia grimesii*, *Pseudomonas putida*, *Alcaligenes faecalis* and *Brevundimonas diminuta*.

Analysis of antagonistic activity of newly isolated strains against phytopatogenic *Fusarium* sp. showed that 18 Gram positive and 6 Gram negative isolates strongly suppressed the growth of this microscopic fungus. Thirteen out of 18 Gram positive bacteria were identified as *B. subtilis*.

We selected two bacterial isolates with highest antagonistic activity against *Fusarium* sp., GM2 and GM5, for further studies. Both strains were gram-positive endospore-forming rods. Oxidase and catalase reactions were positive for

both strains.

The identification by Biotyper (MALDI-TOFF analysis) allowed assigning both strains to the *Bacillus* genus: score value for the strain GM2 was 2.150, and for GM5 – 2.164. This assignment was further confirmed by 16S rRNA and subunit B of gyrase genes homology. Based on the analysis of 16S rRNA full-gene sequence strain GM2 had the closest similarity to *B. subtilis* strains JCM 1465, NBRC 13719 and BCRC 10255 (99% of identity to the sequence of the available 1044 bp fragment of 16S rRNA gene). Sequence of the subunit B of gyrase gene was 97% identical to the corresponding gene from *B. subtilis* subsp. spizizenii TU-B-10 (1011 bp). On the basis of 16S rRNA gene homology strain GM5 was close to the strain *B. subtilis* 168 (98% for sequence with the size of 1010 bp), and also to strains JCM 1465, NBRC 13719 and BCRC 10255 (98% of homology for 942 bp fragment). By the homology of subunit B of DNA gyrase gene sequence the strain GM5 was also similar to the corresponding gene from *B. subtilis* strains TO-A JPC, KCTC 1028 and 168 (98% for 978 bp fragment). Thus, we concluded that both isolates are the new strains of *B. subtilis*.

As shown in Figure 1, the strains *B. subtilis* GM2 and *B. subtilis* GM5 demonstrated antagonistic activity against all tested phytopathogenic fungi: *F. ave-*

Figure 1. Antagonism of *B. subtilis* GM2 and *B. subtilis* GM5 against fungal phytopathogens: (1) *Alternaria alternata* TP 712; (2) *Alternaria solani* 12RKL15; (3) *Alternaria tenuissima;* (4) *Fusarium avenaceum*; (5) *Fusarium solani*; (6) *Fusarium oxysporum*; (7) *Fusarium* sp.; (8) *Fusarium redolens*; (9) *Colletotrichum coccodes* 14raKK6; (10) *Doratomyces* sp. 14raKKLD. Growth inhibition of fungal mycelia was examined in dual culture assay on agar plate.

naceum, *F. oxysporum*, *F. redolens*, *F. solani*, *Fusarium* sp., *A. alternata* TP 712, *A. solani* 12RKL15, *A. tenuissima*, *Doratomyces* sp. 14raKKLD and *C. coccodes* 14raKK6.

Antagonistic activity was evaluated based on the ability to inhibit growth of micromycetes colony compared to the control plates (Table 2). The highest antagonistic activities of both strains were observed against *Doratomyces* sp. 14raKKLD (GM2—72% and GM5—79%). *B. subtilis* GM5 had a greater ability to inhibit growth of all listed micromycetes. For example, the strain GM5 inhibited growth of *A. alternata* TP 712, *F. avenaceum*, *F. redolens* by 72%, 66% and 65%, respectively while GM2 strain inhibited growth of the corresponding fungi by 48%, 54% and 55% respectively.

Next, we tested the ability of bacterial metabolites to inhibit the growth of *F. redolens* in liquid culture. Growth inhibition of micromycetes in the Czapek broth containing bacillary metabolites (20% of the volume) was calculated by the comparison of amount of micromycetes' dry biomass with the control cultures grown in media without metabolite addition (Table 3).

To study the effects of heat-treatment on stability of metabolites autoclaved conditioned medium was used. Metabolites of both strains effectively inhibited growth of micromycetes in the liquid medium (by 82% and 88% for GM2 and

Table 2. Inhibition of mycelial growth by *B. subtilis* GM2 and *B. subtilis* GM5 after seven days of co-culture.

Micromycetes	% of inhibition at 7 days	
	B. subtilis GM2	*B. subtilis* GM5
Alternaria alternate TP 712	48.5 ± 2.4	72.7 ± 4.2
Alternaria solani 12RKL15	45.5 ± 3.1	51.5 ± 2.8
Alternaria tenuissima	45.7 ± 2.6	60.0 ± 4.7
Colletotrichum coccodes 14raKK6	53.3 ± 4.0	63.3 ± 4.4
Doratomyces sp. 14raKKLD	72.4 ± 4.8	79.3 ± 4.1
Fusarium spp.	59.5 ± 3.3	64.9 ± 4.6
Fusarium avenaceum	54.8 ± 3.7	66.7 ± 3.9
Fusarium oxysporum	43.2 ± 3.5	54.1 ± 3.8
Fusarium redolens	55.3 ± 4.0	65.8 ± 3.9
Fusarium solani	44.2 ± 3.3	58.1 ± 3.8

Table 3. Effect of *B. subtilis* GM2 and *B. subtilis* GM5 metabolites on the growth of *F. redolans.*

Strains	% of inhibition	
	No heat treatment	Autoclaved liquid culture (121˚C, 30 min)
B. subtilis GM 2	82.1 ± 4.7	80.6 ± 3.9
B. subtilis GM 5	88.1 ± 3.6	86.6 ± 3.8

GM5 strains, respectively). Autoclaving of the conditioned media did not lead to the loss of inhibitory activity (89% of inhibition by the GM5 strain and 80% by GM2 strain). Therefore, the autoclaving of conditioned media did not cause inactivation of metabolites with antifungal activity.

Thus, the strains *B. subtilis* GM2 and *B. subtilis* GM5 secrete the metabolites into the growth media, which are capable micromycetes' growth inhibition both in liquid and solid media.

The *B. subtilis* GM2 and GM5 strains were analyzed for their ability to produce hydrolytic enzymes (Figure 2) and other antimicrobial metabolites (Table 4).

Both strains had extracellular protease, amylase, pectinase and cellulase activities. Also both strains were able to produce such antimicrobial metabolites as ammonium and HCN. Interestingly, only GM2 strain was capable to secrete siderophores on chrome azurol S (CAS) agar, which was revealed by orange halo formation around colonies after 5 - 7 days of incubation. There was almost no orange halo around colonies of GM5 strain even after 7 days of incubation, which indicates an absence or very low level of siderophore production under growth conditions used in our study (data not shown).

The exometabolites of *B. subtilis* GM2 and *B. subtilis* GM5 affected mycelium morphology of *Fusarium* sp. and *A. alternata* in different ways. In addition to

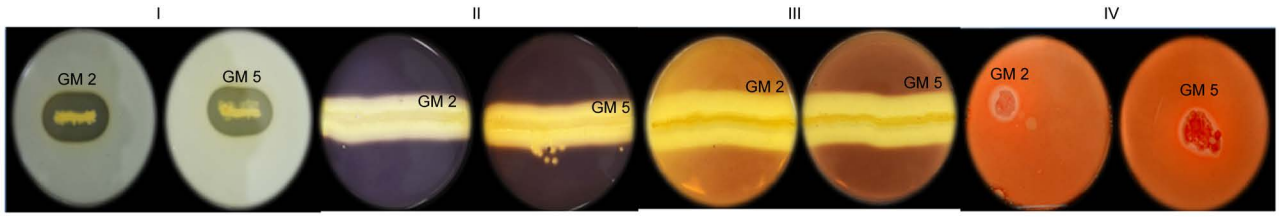

Figure 2. Production of hydrolytic enzymes by *B. subtilis* GM2 and GM5: I—protease production on skim milk agar, II—amylase production on medium containing 0.2% soluble starch, III—pectinase production on medium containing pectin 0.5%, IV—cellulose production on medium containing 0.5% carboxymetyl cellulose.

Table 4. Production of antifungal metabolites by antagonistic *Bacillus subtilis* strains.

Secreted products	*B. subtilis* strains	
	GM2	GM5
siderophore	+	–
ammonia	+	+
HCN	+	+
Hydrolytic enzymes		
protease	+	+
amylase	+	+
pectinase	+	+
cellulase	+	+

*+: positive reaction; –: negative reaction.

the effective inhibition of micromycetes' growth the secreted metabolites of *B. subtilis* GM5 also cause considerable alterations in mycelium morphology (Figure 3).

There were numerous changes in mycelium morphology noticed in the presence of GM5 strain comparing to the control. Specifically, irregular, distorted, wrinkled or swollen regions were present, as well as there were "swollen" cells that reminded chlamydospores. In the presence of *B. subtilis* GM2 strain only single round chlamydospores-like cells were detected. No spore formation was noticed in *Fusarium* sp. grown in co-culture with either of the two *B. subtilis* strains.

We amplified the genes responsible for biosynthesis of various antimicrobial peptides (AMPs) from the strains' genomes: *ituC* (iturin A synthetase C), *bmyB* (bacillomycin L synthetase B), *fenD* (fengycin synthetase), *srfA* (surfactin synthetase subunit 1), *bacA* (bacilysin biosynthesis protein BacA) by the PCR.

As shown on Figure 4, the presence of specific PCR products as single bands of the expected size indicated the presence of corresponding genes in the genomes of studied strains.

Four out of five genes (*ituC*, *bmyB*, *bacA* and *srfA*) were detected in the genome of GM2, and only two genes (*srfA* and *fenD*) were identified in the genome of strain GM5. Thus, the strain GM2 has a potential ability to synthesize cyclic lipopeptides iturin A, bacillomycin L (belonging to the family of iturin), surfactin, and bacilysin (dipeptide antibiotic). The strain GM5 has a potential to synthesize cyclic antibiotics fengycin and surfactin.

To confirm that lipopeptides play a major role in antifungal activity of *B. subtilis* isolates we purified lipopeptide fractions from both strains and used them to

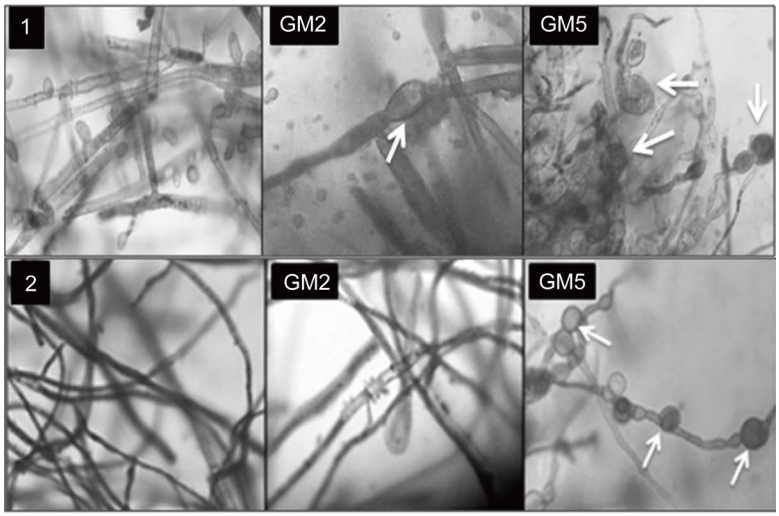

Figure 3. Morphological changes of mycelia of plant pathogenic fungi upon interaction with *B. subtilis* GM2 and *B. subtilis* GM5 in dual culture plates. Images 1 and 2 representative of normal mycelia of *Fusarium* sp. (1) and *A. alternata* (2). GM2 and GM5: mycelia from co-inoculated with *B. subtilis* GM2 and *B. subtilis* GM5. Abnormal conidia germination in presence bacterial isolates. The arrows indicate the formation of swollen chlamydospores-like cells (optical microscopy at 40×).

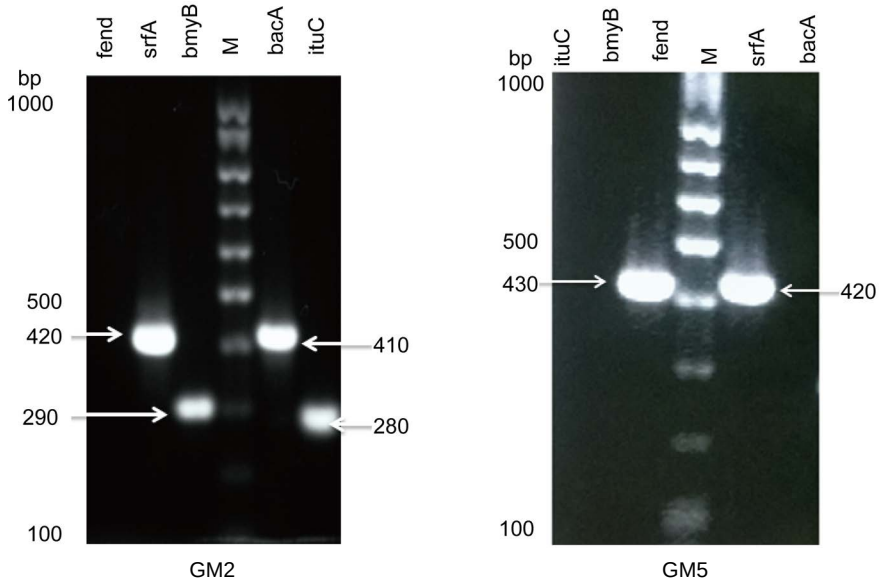

Figure 4. Amplification products of AMP genes. Lane M is a molecular weight marker; *ituC* (iturin A synthetase C), *bmyB* (bacillomycin L synthetase B), *fenD* (fengycin synthetase), *srfA* (surfactin synthetase subunit 1), *bacA* (bacilysin biosynthesis protein BacA).

inhibit growth of *F. solani* and *F. oxisporum*. We found that lipopeptides from *B. subtilis* GM5 had higher antifungal activity compared to the corresponding fraction from *B. subtilis* GM2. Furthermore, lipopeptides from both strains inhibited growth of *F. solani* (**Figure 5**) more efficiently than the growth of *F. oxisporum* (data not shown).

Thus, antagonistic activity of *B. subtilis* GM5 against phytopathogenic fungus *F. solani* is at least partly due to production of lipopeptides by this strain. Despite the presence of wider repertoire of genes encoding for cyclic lipopeptides in the genome of *B. subtilis* GM2 strain (**Figure 4**), HPLC-purified lipopeptide fraction isolated from this strain was less active against *F. solani* compared to a similar fraction isolated from *B. subtilis* GM5 strain (**Figure 5**).

Finally, we used wheat grains to evaluate plant promoting potential of *B. subtilis* GM2 and GM5 strains and to directly compare their ability to protect wheat against *F. oxysporum*.

We found that *B. subtilis* GM2 treatment stimulated both wheat seedling survival and roots and shoots formation. Curiously, *B. subtilis* GM5 did not show any plant growth promoting properties (**Table 5**).

As expected, *F. oxysporum* treatment severely affected both wheat seedling survival and roots and shoots formation. Infected seedlings were covered in *F. oxysporum* mycelium. Treatment of *Fusarium*-infected wheat seedlings with *B. subtilis* GM5 rescued seedling survival to the level of control group and significantly improved roots and shoots formation. In contrast, no protection was observed in the *Fusarium*-infected group, additionally treated with *B. subtilis* GM2 (**Table 5**). Similar results were obtained in the experiments with the tomato plants (data not shown).

Figure 5. Antifungicidal activity of purified lipopeptide fractions. HPLC-purified lipopeptide fractions were tested for their ability to inhibit growth of *F. solani*: 1—methanol-PBS (50/50); 2, 3—lipopeptides purified from conditioned media of *B. subtilis* GM2 (2) and GM5 (3), respectively.

Table 5. Comparative analysis of growth-promoting and antifungal properties of *Bacillus subtilis* isolates.

Treatment	Seedling survival, %	Dry weight, %	
		Roots	Shoots
Control	45.0* ± 10.0	100**	100
B. subtilis GM2	65.0 ± 5.0	132.0 ± 8.5	140.0 ± 6.4
B. subtilis GM5	35.0 ± 5.0	94.0 ± 5.8	95.0 ± 7.6
F. oxysporum	20.0 ± 5.0	7.5 ± 3.5	19.0 ± 4.4
F. oxysporum/*B. subtilis* GM2	10.0 ± 5.0	4.0 ± 2.2	11.5 ± 3.6
F. oxysporum/*B. subtilis* GM5	55.0 ± 5.0	61.0 ± 8.4	94.0 ± 9.3

*The total number of wheat seedlings used in each experiment was taken for 100%; **The average dry weight of roots and shoots in the control group was taken for 100%.

Thus, only *B. subtilis* strain GM5 protected plants against *F. oxysporum in vivo*. Studies to fully evaluate its potential are currently underway.

4. Discussion

The use of beneficial microorganisms in agriculture and other distorted ecosystems can help to protect crops against phytopathogens. It is known that microorganisms associated with plants can promote their growth and development, e.g. due to the growth inhibition of phytopathogenic microorganism [3] [5]. Isolation of new antagonistic strains is necessary to improve biological control methods and restrain plant diseases. Several *Bacillus* strains have a suppressive effect on growth of certain phytopathogens and could be used as biocontrol agents [5] [8] [7]. Some members of *Bacillus* genus have high potential for usage as biological control agents against various plants diseases (Kumar *et al*. 2012). For instance, *B. subtilis* strains have demonstrated antagonistic activity against

Fusarium sp. [10] [26].

In this study, two strains with high antagonistic activity against micromycetes were isolated from potato's rhizosphere.

MALDI-TOF analysis assigned isolated strains GM2 and GM5 to *Bacillus* genus and predicted that they belong to *B. subtilis* species. An accurate species assignment of isolates using this technique could be done when the Score values are in the range 2.3 - 3.0. Further studies based on 16S rRNA and gyrase subunit B genes homology confirmed that isolated bacteria represent new strains of *B. subtilis*.

It is known that *B. subtilis* species are heterogeneous both phenotypically and genotypically [27]. High genetic heterogeneity of different *Bacillus* species [5] [28] particularly *B. subtilis* allows to suggest that search and identification of new strains from different sources may expand the number of practically important strains and to improve our understanding of mechanisms involved in antagonistic interactions.

Comparative analysis of antagonistic activity of isolated *B. subtilis* isolates showed that strain GM5 inhibits the growth of various micromycetes more effectively than the strain GM2 especially on solid media. The inhibitory activity of extracellular metabolites was not affected by sterilization of *Bacillus*-conditioned media by autoclaving, which suggests that the main antifungal activity is associated with thermo-stable metabolites. It is known that *Bacillus* ssp. protect plants against phytopathogenic bacteria and micromycetes through a number of mechanisms, in particular through the synthesis of different cyclic lipopeptides with inhibitory activity against phytopathogens [29]. The genes responsible for biosynthesis of antimicrobial peptides were identified in different species of *Bacillus*, and mostly in *B. subtilis* and *B. amyloliquefaciens* strains [29]. The analysis of large number of *Bacillus* spp. isolates (184) associated with plants showed that the significant number of strains possessed at least 2 to 4 genes encoding for antimicrobial peptides. Majority of strains carry *srfA*, *bacA*, *bmyB*, *fenD* genes [30]. Thus, pre-screening for the presence of these genes could be useful for selection of potentially promising strains for biocontrol of phytopathogens.

We have shown that two newly isolated strains of *B. subtilis* differ in the repertoire of genes encoding for antimicrobial peptides. Cyclic lipopeptides (CLP) produced by *B. subtilis* strains have been shown to protect host plants from a numbers of pathogens. CLP produced by *B. subtilis* can be grouped into three families: surfactins, fengycins, and iturins, each of which has shown different antimicrobial characteristics [9]. PCR-screening revealed that the strain GM2 contains four genes responsible for synthesis of cyclic lipopeptides like iturin A, bacillomycin L (belongs to the iturin family) and surfactin and also dipeptide antibiotic bacilysin. The strain GM5 has two genes necessary for synthesis of cyclic lipopeptides surfactin and fengycin. Higher antagonistic activity of GM5 correlated with the presence of fengycin gene that was not identified in GM2 strain. Moreover, purified lipopeptide fraction from GM5 strain showed significantly higher ability to inhibit fungal growth compare to a similar fraction purified

from GM2 strain. Fengycins are cyclic lipodecapeptides which specifically inhibit growth of filamentous fungi [31]. Fengycin draws a lot of attention in the recent years because of its lower hemolytic activity compared to other *B. subtilis* lipopeptides and its strong antifungal capability, specifically against filamentous fungi [32]. Fengycin is known to affect the biological membranes of fungi, causing membrane pore formation, however, the precise mechanism of fengycin's action and the intrinsic biochemical determinants implicated in its antifungal activity have not been fully elucidated [31]. Simultaneous production of fengycins and surfactins could be important for the efficiency of *F. graminearum* control by strain *B. subtilis* SG6 [26].

There were significant changes in the mycelium morphology caused by bacteria in comparison to the control: irregular, distorted, shrunken or swollen areas appeared; many "swollen" chlamydospore-like cells were seen. The appearance of such anomalous formations in mycelium in the presence of metabolites of bacterial antagonists was frequently noticed by others [33] [34]. Antifungal action of *Bacillus* metabolites may be due to disruption of fungal cell wall and inhibition of normal conidia development. It is known that chlamydospores with thick walls are developed by the modification of hyphal and conidial cells under unfavorable environmental conditions, such as at low temperature, and these resting bodies act as primary inocula in subsequent soil-borne infections, whereas the microconidia and macroconidia formed by *F. oxysporum* are important in secondary infection [35] [36]. Minerdi *et al.* [37] reported that microbial symbionts silence the virulence of *F. oxysporum* and that changes in cell morphogenesis by *F. oxysporum* underlie this suppression.

Various bacterial features were examined to identify other secondary metabolites and additional factors which may correlate the differences in antagonistic activity of studied strains. It is known that extracellular hydrolases make important contributions to the antimicrobial potential of antagonists. It was shown that synthesis of extracellular hydrolases correlates with antifungal potential because some hydrolytic enzymes are involved in the degradation of phytopathogenic micromycetes' cell wall [10] [38]. It was also suggested that pectinases and amylases contribute to the colonization of roots by bacteria and therefore may play an important role in plant growth stimulation [28]. We have shown that both strains secrete proteinases, amylases, pectinases and cellulases to the conditioned media.

We have found that out of two *B. subtilis* strains only one of them, GM2, produced significant amount of siderophores as indicated by formation of orange halos around the colonies on the CAS agar medium. It was suggested that the synthesis of siderophores is associated with growth-promoting and antagonistic activity against phytopathogens [3]. In the soil, ability to produce siderophores plays a central role in the capacity of different bacteria to promote plant development due to increasing the iron absorption by plants and defense of plants from toxic metals such as nickel and cadmium [39] [40]. The antagonism against pathogenic bacteria and fungi is based on the iron binding by sidero-

phore-producing bacteria that limits iron availability for other microorganisms and primarily for phytopathogenic micromycetes [41]. Both strains produce cyanides and ammonium that could contribute to their antifungal activity. It is known that cyanogens produced by *Bacillus* can inhibit fungal growth [4].

Nevertheless, our findings showed that the ability to secrete highly active proteinases and to produce siderophores does not correlate directly with the antifungal potential of the *Bacillus* strains. While siderophore-producing strain GM2 expressed plant growth promoting properties, only *B. subtilis* GM5 strain was able to protect wheat seedlings and tomato plants against *F. oxysporum*, an important pathogen of wheat [42] [43] and tomato [44].

5. Conclusion

We have isolated two new strains of *B. subtilis* that differ in their antagonistic activity against a number of phytopathogenic fungi. Only *B. subtilis* GM5 strain was able to protect wheat from phytopathogenic fungus *Fusarium oxysporum in vivo.* The antifungal activity of GM5 strain was attributed with secretion of antimicrobial peptides. We suggest that a high antifungal activity might be associated with the presence of *fenD* gene identified only in GM5 strain. Thus, *B. subtilis* GM5 can find application in agriculture as bioinoculant for agriculturally important plants.

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Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

References

[1] Berg, G., Fritze, A., Roskot, N. and Smalla, K. (2001) Evaluation of Potential Biocontrol Rhizobacteria from Different Host Plants of *Verticillium dahliae* Kleb. *Journal of Applied Microbiology*, **91**, 963-971. https://doi.org/10.1046/j.1365-2672.2001.01462.x

[2] Cazorla, F.M., Romero, D., Perez-Garcia, A., Lugtenberg, B.J., Vicente, A.D. and Bloemberg, G. (2007) Isolation and Characterization Of antagonistic *Bacillus subtilis* Strains from the Avocado Rhizoplane Displaying Biocontrol Activity. *Journal of Applied Microbiology*, **103**, 1950-1959. https://doi.org/10.1111/j.1365-2672.2007.03433.x

[3] Beneduzi, A., Ambrosini, A. and Passaglia, L.M. (2012) Plant Growth-Promoting

Rhizobacteria (PGPR): The Potential as Antagonists and Biocontrol Agents. *Genetics and Molecular Biology*, **35**, 1044-1051. https://doi.org/10.1590/S1415-47572012000600020

[4] Kumar, P., Dubey, R.C. and Maheshwari, D.K. (2012) *Bacillus* Strains Isolated from Rhizosphere Showed Plant Growth Promoting and Antagonistic Activity against Phytopathogens. *Microbiological Research*, **167**, 493-499. https://doi.org/10.1016/j.micres.2012.05.002

[5] Choudhary, D.K. and Johri, B.N. (2009) Interactions of *Bacillus* spp. and Plants—with Special Reference to Induced Systemic Resistance (ISR). *Microbiological Research*, **164**, 493-513. https://doi.org/10.1016/j.micres.2008.08.007

[6] Pinchuk, I.V., Bressollier, P., Sorokulova, I.B., Verneuil, B. and Urdaci, M.C. (2002) Amicoumacin Antibiotic Production and Genetic Diversity of *Bacillus subtilis* Strains Isolated from Different Habitats. *Research in Microbiology*, **153**, 269-276. https://doi.org/10.1016/S0923-2508(02)01320-7

[7] Wang, S., Wu, H., Qiao, J., Ma, L., Liu, J., Xia, Y. and Gao, X. (2009) Molecular Mechanism of Plant Growth Promotion and Induced Systemic Resistance to Tobacco Mosaic Virus by *Bacillus* spp. *Journal of Microbiology and Biotechnology*, **19**, 1250-1258. https://doi.org/10.4014/jmb.0901.008

[8] Kim, P.I., Ryu, J., Kim, Y.H. and Chi, Y.T. (2010) Production of Biosurfactant Lipopeptides Iturin A., Fengycin, and Surfactin A from *Bacillus subtilis* CMB32 for Control of *Colletotrichum gloeosporioides*. *Journal of Microbiology and Biotechnology*, **20**, 138-145.

[9] Falardeau, J., Wise, C., Novitsky, L. and Avis, T.J. (2013) Ecological and Mechanistic Insights into the Direct and Indirect Antimicrobial Properties of *Bacillus subtilis* lipopeptides on Plant Pathogens. *Journal of Chemical Ecology*, **39**, 869-878. https://doi.org/10.1007/s10886-013-0319-7

[10] Baysal, O., Lai, D., Xu, H., Siragusa, M., Casiskan, M., Carimi, F., Silva, J.A.T. and Tor, M. (2013) A Proteomic Approach Provides New Insights into the Control of Soil-Borne Plant Pathogens by *Bacillus* Species. *PloS One*, **8**, e53182. https://doi.org/10.1371/journal.pone.0053182

[11] Tan, Z., Lin, B. and Zhang, R. (2013) A Novel Antifungal Protein of *Bacillus subtilis* B25. *SpringerPlus*, **2**, 543. https://doi.org/10.1186/2193-1801-2-543

[12] Li, X.Y., Yang, J.J., Mao, Z.C., Ho, H.H., Wu, Y.X. and He, Y.Q. (2014) Enhancement of Biocontrol Activities and Cyclic Lipopeptides Production by Chemical Mutagenesis of *Bacillus subtilis* XF-1, a Biocontrol Agent of *Plasmodiophora brassicae* and *Fusarium solani*. *Indian Journal of Microbiology*, **54**, 476-479. https://doi.org/10.1007/s12088-014-0471-y

[13] Baffoni, L., Gaggia, F., Dalanaj, N., Prodi, A., Nipoti, P., Pisi, A., Biavati, B. and Di Gioia, D. (2015) Microbial Inoculants for the Biocontrol of *Fusarium* spp. in Durum Wheat. *BMC Microbiology*, **15**, 242. https://doi.org/10.1186/s12866-015-0573-7

[14] Shternshis, M.V., Belyaev, A.A., Matchenko, N.S., Shpatova, T.V. and Lelyak, A.A. (2015) Possibility of Biological Control of Primocane Fruiting Raspberry Disease Caused by *Fusarium sambucinum*. *Environmental Science and Pollution Research*, **22**, 15656-15662. https://doi.org/10.1007/s11356-015-4763-5

[15] Guo, Q., Dong, W., Li, S., Lu, X., Wang, P., Zhang, X., Wang, Y. and Ma, P. (2014) Fengycin Produced by *Bacillus subtilis* NCD-2 Plays a Major Role in Biocontrol of Cotton Seedling Damping-Off Disease. *Microbiological Research*, **169**, 533-540. https://doi.org/10.1016/j.micres.2013.12.001

[16] Zhang, C.X., Zhao, X., Han, F., Yang, M.F., Chen, H., Chida, T. and Shen, S.H.

(2009) Comparative Proteome Analysis of Two Antagonist *Bacillus subtilis* Strains. *Journal of Microbiology and Biotechnology*, **19**, 351-357. https://doi.org/10.4014/jmb.0805.346

[17] Wu, L., Wu, H.J., Qiao, J., Gao, X. and Borriss, R. (2015) Novel Routes for Improving Biocontrol Activity of *Bacillus* Based Bioinoculants. *Frontiers in Microbiology*, **6**, 1395. https://doi.org/10.3389/fmicb.2015.01395

[18] Sambrook, J. and Russel, D.W. (2001) Molecular Cloning: A Laboratory Manual. 3rd Edition, Cold Spring Harbor Laboratory Press, New York.

[19] Mullis, K.B. and Faloona, F.A. (1987) Specific Synthesis of DNA *in Vitro* via a Polymerase-Catalyzed Chain Reaction. *Methods in Enzymology*, **155**, 335-350. https://doi.org/10.1016/0076-6879(87)55023-6

[20] Raper, K.B. and Fennell, D.I. (1965) The Genus *Aspergillus*. Williams and Wilkins, Philadelphia, 686 p.

[21] Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A. and Smalla, K. (2002) Plant-Dependent Genotypic and Phenotypic Diversity of Antagonistic Rhizobacteria Isolated from Different *Verticillium* Host Plants. *Applied and Environmental Microbiology*, **68**, 3328-3338. https://doi.org/10.1128/AEM.68.7.3328-3338.2002

[22] Baldan, E., Nigris, S., Romualdi, C., D'Alessandro, S., Clocchiatti, A., Zottini, M., Stevanato, P., Squartini, A. and Baldan, B. (2015) Beneficial Bacteria Isolated from Grapevine Inner Tissues Shape *Arabidopsis thaliana* Roots. *PLoS ONE*, **10**, e0140252. https://doi.org/10.1371/journal.pone.0140252

[23] Nagarajkumar, M., Bhaskaran, R. and Velazhahan, R. (2004) Involvement of Secondary Metabolites and Extracellular Lytic Enzymes Produced by *Pseudomonas fluorescens* in Inhibition of *Rhizoctonia solani*, the Rice Sheath Blight Pathogen. *Microbiological Research*, **159**, 73-81. https://doi.org/10.1016/j.micres.2004.01.005

[24] Trivedi, P., Pandey, A. and Palni, L.M. (2008) *In Vitro* Evaluation of Antagonistic Properties of *Pseudomonas corrugata*. *Microbiological Research*, **163**, 329-336. https://doi.org/10.1016/j.micres.2006.06.007

[25] Bakker A.W. and Schippers, B. (1987) Microbial Cyanide Production in the Rhizosphere in Relation to Potato Yield Reduction and *Pseudomonas* SPP-Mediated Plant Growth-Stimulation. *Soil Biology and Biochemistry*, **19**, 451-457. https://doi.org/10.1016/0038-0717(87)90037-X

[26] Roy, A., Mahata, D., Paul, D., Korpole, S., Franco, O.L. and Mandal, S.M. (2013) Purification, Biochemical Characterization and Self-Assembled Structure of a Fengycin-Like Antifungal Peptide from *Bacillus thuringiensis* Strain SM1. *Frontiers in Microbiology*, **4**, 332. https://doi.org/10.3389/fmicb.2013.00332

[27] Zhao, Y., Selvaraj, J.N., Xing, F., Zhou, L., Wang, Y., Song, H., Tan, X., Sun, L., Sangare, L., Folly, Y.M. and Liu, Y. (2014) Antagonistic Action of *Bacillus subtilis* Strains SG6 on *Fusarium graminearum*. *PLoS ONE*, **9**, e92486. https://doi.org/10.1371/journal.pone.0092486

[28] Kopac, S., Wang, Z., Wiedenbeck, J., Sherry, J., Wu, M. and Cohan, F.M. (2014) Genomic Heterogeneity and Ecological Speciation within One Subspecies of *Bacillus subtilis*. *Applied and Environmental Microbiology*, **80**, 4842-4853. https://doi.org/10.1128/AEM.00576-14

[29] Hossain, M.J., Ran, C., Liu, K., Ryu, C.M., Rasmussen-Ivey, C.R., Williams, M.A., Hassan, M.K., Choi, S.K., Jeong, H., Newman, M., Kloepper, J.W. and Liles, M.R. (2015) Deciphering the Conserved Genetic Loci Implicated in Plant Disease Control through Comparative Genomics of *Bacillus amyloliquefaciens* subsp. Plantarum. *Frontiers in Plant Science*, **6**, 631. https://doi.org/10.3389/fpls.2015.00631

[30] Mora, I., Cabrefiga, J. and Montesinos, E. (2015) Cyclic Lipopeptide Biosynthetic

Genes and Products, and Inhibitory Activity of Plant-Associated *Bacillus* against Phytopathogenic Bacteria. *PLoS ONE*, **10**, e0127738. https://doi.org/10.1371/journal.pone.0127738

[31] Mora, I., Cabrefiga, J. and Montesinos, E. (2011) Antimicrobial Peptide Genes in *Bacillus* strains from Plant Environments. *International Microbiology*, **14**, 213-223.

[32] Wise, C., Falardeau, J., Hagberg, I. and Avis, T.J. (2014) Cellular Lipid Composition Affects Sensitivity of Plant Pathogens to Fengycin, an Antifungal Compound Produced by *Bacillus subtilis* Strain CU12. *Phytopathology*, **104**, 1036-1041. https://doi.org/10.1094/PHYTO-12-13-0336-R

[33] Deleu, M., Paquot, M., and Nylander, T. (2008) Effect of Fengycin, a Lipopeptide Produced by *Bacillus subtilis*, on Model Biomembranes. *Biophysical Journal*, **94**, 2667-2679. https://doi.org/10.1529/biophysj.107.114090

[34] Petatan-Sagahon, I., Anducho-Reyes, M.A., Silva-Rojas, H.V., Arana-Cuenca, A., Tellez-Jurado, A., Cardenas-Alvarez, I.O. and Mercado-Flores, Y. (2011) Isolation of Bacteria with Antifungal Activity against the Phytopathogenic Fungi *Stenocarpella maydis* and *Stenocarpella macrospora*. *International Journal of Molecular Sciences*, **12**, 5522-5537. https://doi.org/10.3390/ijms12095522

[35] Ohara T. and Tsuge, T. (2004) FoSTUA, Encoding a Basic Helix-Loop-Helix Protein, Differentially Regulates Development of Three Kinds of Asexual Spores, Macroconidia, Microconidia, and Chlamydospores, in the Fungal Plant Pathogen *Fusarium oxysporum*. *Eukaryotic Cell*, **3**, 1412-1422. https://doi.org/10.1128/EC.3.6.1412-1422.2004

[36] Fujiwara, K., Iida, Y., Iwai, T., Aoyama, C., Inukai, R., Ando, A., Oqawa, J., Ohnishi, J., Terami, F., Takano, M. and Shinohara, M. (2013) The Rhizosphere Microbial Community in a Multiple Parallel Mineralization System Suppresses the Pathogenic Fungus *Fusarium oxysporum*. *Microbiology Open*, **2**, 997-1009. https://doi.org/10.1002/mbo3.140

[37] Minerdi, D., Moretti, M., Gilardi, G., Barberio, C., Gullino, M.L. and Garibaldi, A. (2008) Bacterial Ectosymbionts and Virulence Silencing in a *Fusarium oxysporum* Strain. *Environmental Microbiology*, **10**, 1725-1741. https://doi.org/10.1111/j.1462-2920.2008.01594.x

[38] Wu, Y., Yuan, J., Raza, W., Shen, Q. and Huang, Q. (2014) Biocontrol Traits and Antagonistic Potential of *Bacillus amyloliquefaciens* Strain NJZJSB3 against *Sclerotinia sclerotiorum*, a Causal Agent of Canola Stem Rot. *Journal of Microbiology and Biotechnology*, **24**, 1327-1336. https://doi.org/10.4014/jmb.1402.02061

[39] Dimkpa, C.O., Merten, D., Svatos, A., Büchel, G. and Kothe, E. (2009) Siderophores Mediate Reduced and Increased Uptake of Cadmium by *Streptomyces tendae* F4 and Sunflower (*Helianthus annuus*), Respectively. *Journal of Applied Microbiology*, **107**, 1687-1696. https://doi.org/10.1111/j.1365-2672.2009.04355.x

[40] Berendsen, R.L., van Verk, M.C., Stringlis, I.A., Zamioudis, C., Tommassen, J., Pieterse, C.M and Bakker, P.A. (2015) Unearthing the Genomes of Plant-Beneficial *Pseudomonas* Model Strains WCS358, WCS374 and WCS417. *BMC Genomics*, **16**, 539. https://doi.org/10.1186/s12864-015-1632-z

[41] Pastor, N., Carlier, E., Andres, J., Rosas, S.B. and M. Rovera, M. (2012) Characterization of Rhizosphere Bacteria for Control of Phytopathogenic Fungi of Tomato. *Journal of Environmental Management*, **95**, 5332-5337. https://doi.org/10.1016/j.jenvman.2011.03.037

[42] Ioos, R., Belhadj, A. and Menez, M. (2004) Occurrence and Distribution of *Microdochium nivale* and *Fusarium* Species Isolated from Barley, Durum and Soft Wheat Grains in France from 2000 to 2002. *Mycopathologia*, **158**, 351-362.

https://doi.org/10.1007/s11046-004-2228-3

[43] Treikale, O., Priekule, I., Javoisha, B. and Lazareva, L. (2010) *Fusarium* Head Blight: Distribution in Wheat in Latvia. *Communications in Agricultural and Applied Biological Sciences*, **75**, 627-634.

[44] Takken F. and Rep, M. (2010) The Arms Race between Tomato and *Fusarium oxysporum*. *Molecular Plant Pathology*, **11**, 309-314. https://doi.org/10.1111/j.1364-3703.2009.00605.x