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Isolation of *Bacillus subtilis* strain SEM-2 from silkworm excrement and characterisation of its antagonistic effect against *Fusarium* spp.

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Abstract Fusarium wilt is a devastating soil-borne disease mainly caused by highly host-specific *formae speciales* of *Fusarium* spp. Antagonistic microorganisms play a very important role in Fusarium wilt control. Isolation of potential biocontrol strains has become increasingly important. Bacterial strain SEM-2 was isolated from the high-temperature stage of silkworm excrement composting. SEM-2 exhibited a considerable antagonistic effect against *Fusarium graminearum* mycelial growth and spore germination. The results of pot experiments suggested that SEM-2 has a better inhibitory effect on the early stage of disease occurrence. The GFP-labelled SEM-2 coated on the surface of tomato seeds colonised the roots of tomato plants in 15 days. Genome sequencing identified SEM-2 as a new strain of *Bacillus subtilis*, and genome annotation and analysis determined gene clusters related to the biosynthesis of antimicrobials, such as bacillaene, fengycin, bacillibactin, subtilosin A, surfactin and bacilysin. Interestingly, liquid chromatography-quadrupole-time of flight-mass spectrometry revealed that metabolites in pathways associated with the synthesis of secondary metabolites and antibiotics were highly differentially expressed. These findings may help to explain the mode-of-action of *B. subtilis* SEM-2 against *Fusarium* spp.

Keywords: Species characterisation; Sequence analysis; Biocontrol Agent; Silkworm excrement

Running title: Isolation and characterisation of *Bacillus subtilis* strain SEM-2
**Introduction**

Fusarium wilt is a devastating soil-borne disease mainly caused by highly host-specific *formae speciales* of *Fusarium* spp., which affects at least 100 different cultivated plants (Fourie et al. 2011). *Fusarium* spp. can infect and destroy the vascular bundle system of the host plant and produce toxins in the process of growth, development and metabolism, which causes crop wilting and death, affecting the yield and quality of the plant (Cosic et al. 2012; Choi et al. 2013; Scherm et al. 2013). In the past, prevention and management methods against *Fusarium* wilt had mainly depended on chemical-based pesticides. However, long-term use of fungicides can lead to fungicide resistance in the pathogen, environmental pollution, pesticide residual effects on human health as well as other serious ecological and agricultural safety issues (Haas and Defago 2005; Veliz et al. 2017). Consequently, researchers began to investigate the use of alternative synthetic agrochemicals, which led to the development of biopesticides (Pal et al. 2006; Seiber et al. 2014; Bardin et al. 2015).

Biological control of plant diseases is a method to effectively control crop diseases by using beneficial microorganisms and microbial metabolites. There are various biocontrol factors for biological control of plant diseases, including antagonistic microorganisms, antibiotics and plant elicitors. At present, most of the biocontrol bacteria used in the prevention and management of plant diseases include *Bacillus* spp., *Pseudomonas* spp., *Agrobacterium* spp. or *Pasteurella* spp. (Ferreira et al. 1991; Law et al. 2017). Many different *Bacillus* spp., such as *Bacillus subtilis*, *B. amylolytica*, *B. mega* and *B. brevis*, have bio-promoting and disease-preventing functions (Agarwal et al. 2017; Bapat and Shah 2000; Du et al. 2017; Huang et al. 2016; Jiang et al. 2017; Midhun et al. 2017; Raza et al. 2016; Xu et al. 2014).

*B. subtilis* occupies an important position in the microbial fertiliser and pesticide market owing to
its remarkable bio-promoting and disease-controlling effects (Zhao et al. 2015; Zheng et al. 2019). There have been many reports indicating that B. subtilis strains can prevent and control fungal and bacterial diseases of different plants, such as rice sheath blight, rice blast, wheat sheath blight, tomato leaf mould, cucumber wilt, and others. Daivasikamani and Rajanaika (2009) used B. subtilis to control Hemileia vastatrix, a pathogen of coffee rust (Daivasikamani and Rajanaika 2009), while QST713 powder has been reported to inhibit Fusarium wilt, scab, grey mould and some fungal diseases (Omur et al. 2008). In China, the use of B. subtilis to control plant diseases has also made significant progress. B. subtilis B29 has strong antagonistic effects on Fusarium spp., Pythium spp. and Rhizoctonia solani, the causative agents of soybean root rot, and can increase plant yield (Li et al. 2008). In addition, B. subtilis b916, developed by the Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, China, had also been registered as a pesticide. The field control effect of B. subtilis B916 on rice sheath blight was 50%–80% for 10 consecutive years (Li et al. 2007). According to statistics reported in May 2019, more than 60% of microbial fertiliser products registered by the Ministry of Agriculture of China contained B. subtilis (http://www.biofertilizer 95.cn/zhdjcpml). Therefore, B. subtilis plays an important role as a microbial fertiliser and biopesticide. In this study, strain SEM-2 was isolated from silkworm excrement during fermentation at the high-temperature stage, and was found to have a significant antagonistic effect on Fusarium spp. and Fusarium wilt. Classification and functional analyses were performed, which clearly demonstrated the significant biocontrol potential of strain SEM-2.

**Materials and Methods**
Silkworm excrement was sampled during high-temperature composting fermentation at the Guangdong Academy of Agricultural Sciences (GAAS), GuangZhou, China. A total of 5 g of silkworm excrement was added into a triangular bottle containing 45 mL of sterile water and 10 sterile glass beads and oscillated for 20 min. Strain isolation was conducted by plating 10-fold dilutions of the mixture on inorganic phosphorous selective medium (Li et al. 2019). Individual colonies were re-streaked onto fresh nutrient agar (NA) slants, and pure cultures were further sub-cultured for morphological and molecular analyses.

Antagonism of strain SEM-2 against Fusarium spp.

Plugs of four plant-pathogenic fungi, namely, Fusarium catenulatum, F. oxysporum, F. solani and F. graminearum, provided by the Sericulture and Agri-Food Research Institute of GAAS, were inoculated onto potato dextrose agar (PDA) plates, and antagonism and mycelial morphological analyses were conducted using scanning electron microscopy (SEM) according to the method described by Li et al. (2019).

Determination of the germination rate of Fusarium spores

Fusarium spp. cells were cultured on PDA plates at 30°C for more than 5 days, and conidia were collected. A conidial suspension (concentration adjusted to $10^6$–$10^7$ CFU/mL with sterile deionised water) was prepared for each Fusarium spp. Cell-free liquid SEM-2 culture filtrate was freeze-dried, and re-suspended solutions (0.1 g freeze-dried culture/mL sterile water) were prepared and mixed with the conidial suspension and sterile water at the ratios of 1:1:0, 1:0.5:0.5 and 1:0.25:0.75. The control (CK) was a mixture of conidial suspension and sterile water at a ratio of 1:1. The suspensions were cultured at...
30°C and 180 rpm, and the number of germinated and non-germinated conidia was determined at the indicated time intervals.

**GFP labelling of strain SEM-2**

Extraction of the pGFP22 vector: The pGFP22 plasmid (Biovector-NTCC, Beijing, China) was extracted from the *B. subtilis* host using Plasmid Extraction Kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. Because of the thicker and denser cell wall of Gram-positive bacteria, 50 mg/mL lysozyme was mixed with solution 1 and shaken at 37°C and 180 rpm for 1 h, with the rest of the steps remaining unchanged.

Preparation and transformation of competent cells of strain SEM-2: A single colony of strain SEM-2 was placed in 5 mL of HS liquid medium comprising 0.2% (NH₄)₂SO₄, 1.4% K₂HPO₄, 0.6% KH₂PO₄, 0.1% sodium citrate, 0.012% MgSO₄, 0.5% (w/v) glucose, 0.005% (w/v) L-tryptophan, 0.02% (w/v) casein, 0.5% (w/v) yeast extract, 0.8% (w/v) arginine and 0.04% histidine, and incubated overnight at 37°C and 250 rpm. Then, 0.5 mL of the overnight culture was inoculated into 50 mL of HS medium and cultured at 37°C and 250 rpm. At the end of the logarithmic growth phase, 1 mL of bacterial culture was mixed with 20 mL of LS medium comprising 0.2% (NH₄)₂SO₄, 1.4% K₂HPO₄, 1.6% KH₂PO₄, 0.1% sodium citrate, 0.012% MgSO₄, 0.012% MgSO₄, 0.5% (w/v) glucose, 0.005% (w/v) L-tryptophan, 0.01% (w/v) casein, 0.1% (w/v) yeast extract, 0.05% MgCl₂ and 0.005% CaCl₂, and cultured slowly for 2 h at 37°C and 100 rpm. Subsequently, 10 μL of EGTA (10 mM) were added to 1 mL of the culture suspension and incubated for 5 min at 37°C and 100 rpm. Then, 0.5 mL of the culture suspension was poured into a tube, and 10–20 μL of plasmid DNA (about 1 μg) were added to the tube and incubated for 90 min at 37°C and 250 rpm. After that, 100 μL of the bacterial culture were plated on
LB agar containing chloramphenicol (10 μg/mL) and incubated overnight at 37°C, and subjected to fluorescence screening.

Colonisation of tomato roots by strain SEM-2

Inorganic salt medium (1.9 g/L KNO₃, 1.65 g/L NH₄NO₃, 0.37 g/L MgSO₄·7H₂O, 0.17 g/L KH₂PO₄, 0.44 g/L CaCl₂·2H₂O, 0.02 g/L MnSO₄·4H₂O, 0.008 g/L ZnSO₄·7H₂O, 0.006 g/L H₃BO₃, 0.8 mg/L KI, 0.25 mg/L Na₂MoO₄·2H₂O, 0.025 mg/L CuSO₄·5H₂O, 0.025 mg/L CoCl₂·6H₂O, 37.3 mg/L Na₂-EDTA, 27.8 mg/L FeSO₄·7H₂O and 3.2 g/L Phytagel; pH 6.6–6.8) was prepared, poured into tissue culture bottles (25 mL/bottle) and sterilised at 121°C for 30 min. Strain SEM-2 was cultured in NB medium overnight, and 0.5 mL of the bacterial suspension (1×10⁸ CFU/mL) was added into the tissue culture bottle under sterile conditions. Subsequently, pre-germinated tomato seeds were added to the tissue culture bottle and incubated. After 15 days, the roots were sampled, washed with sterilised water, submerged in sterilised water for 30 min and assessed by fluorescence microscopy.

Investigation of disease occurrence in tomato seedlings using the pot experiment

Experimental soil was collected from a typical mulberry garden. Two concentrations of Fusarium spores (10⁵ and 10⁷ CFU/mL) were employed, and three concentrations of strain SEM-2-G suspensions (10⁶, 10⁷ and 10⁸ CFU/mL) were prepared. Normal soil from the same source without Fusarium spores and SEM-2-G suspension was used as the control. The tomato seeds were soaked and seeded in pots with different concentrations of Fusarium spores, and SEM-2-G suspension was irrigated at different concentrations around the seeds on the second day after seeding. The experimental design was as follows:
Normal control group (CK): without *Fusarium* spores and strain SEM-2 suspension; Low spore concentration group (L): A *Fusarium* spore suspension at a spore concentration of $10^5$ CFU/mL was mixed with soil. This group was further divided into four sub-groups according to the concentration of SEM-2-G suspension as follows: Blank medium group (LA) = 10 mL of blank medium without strain SEM-2-G addition; $10^6$ CFU/mL treatment group (LB) = addition of 10 mL of SEM-2-G suspension at a concentration of $10^6$ CFU/mL; $10^7$ CFU/mL treatment group (LC) = addition of 10 mL of SEM-2-G suspension at a concentration of $10^7$ CFU/mL; $10^8$ CFU/mL treatment group (LD) = addition of 10 mL of SEM-2-G suspension at a concentration of $10^8$ CFU/mL.

High spore concentration group (H): *Fusarium* spores suspension at a concentration of $10^7$ CFU/mL was mixed with the soil. This group was further divided into four sub-groups according to the concentration of SEM-2-G suspension as follows: Blank medium group (HA): 10 mL of blank medium without strain SEM-2-G addition; $10^6$ CFU/mL treatment group (HB): addition of 10 mL of SEM-2-G suspension at a concentration of $10^6$ CFU/mL; $10^7$ CFU/mL treatment group (HC): addition of 10 mL of SEM-2-G suspension at a concentration of $10^7$ CFU/mL and $10^8$ CFU/mL treatment group (HD): addition of 10 mL of SEM-2-G suspension at a concentration of $10^8$ CFU/mL.

Each treatment consisted of three replicates and each group comprised 10 basins. The tomato seeds were soaked and germinated normally, and then seeded in pots. The potted plants were placed in a greenhouse at room temperature and watered to keep the soil moist. The disease incidence of tomato was investigated on days 7, 14 and 21. The colonisation of strain SEM-2-G was detected by fluorescence microscopy after 21 days.

*Genome sequencing of strain SEM-2*
The genomic DNA (gDNA) of strain SEM-2 was extracted from cells grown in NB medium using miniBEST Bacterial Genomic DNA Extraction Kit (DV810A, TaKaRa) according to the manufacturer’s instructions. Then, the extracted gDNA was quality-assessed and sent to BGI Technology Service Co., Ltd (ShenZhen, China) for genome sequencing.

Raw sequencing data were filtered to remove adapters and low-quality reads to generate clean data. Short Oligonucleotide Analysis Package (SOAPdenovo; www.soap.genomics.org.cn) was used to assemble reads after filtering and perform bioinformatics analysis, including genomic component and gene function analyses. We used Glimmer, rRNAmer and tRNAscan (Delcher et al. 1999; Lagesen et al. 2007; Lowe and Eddy 1997) to predict genes, rRNAs and tRNAs, and their secondary structures, from the assembled results. Functional annotation was accomplished by analysis of protein sequences, and we aligned the obtained gene sequences with database sequences to obtain annotation information. To ensure the results were of biological significance, the highest scoring alignment results were chosen for gene annotation, and functional annotation was achieved by BLAST searching gene sequences against Gene Ontology (GO; http://www.geneontology.org/) (Ashburner et al. 2000), Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) (Kanehisa et al. 2004, 2006) and Clusters of Orthologous Groups of proteins (COG; http://www.ncbi.nlm.nih.gov/COG) databases (Tatusov et al. 1997, 2003). The assembled data were submitted to the GenBank database under submission number NGUK00000000.

Core–Pan gene analysis was also performed. Firstly, genes were acquired from a reference genome as the gene pool. Then, genes predicted from query samples were BLAST-searched against the gene pool, and the BLAST results were filtered based on length and sequence identity. The BLAST coverage ratios (BCR) of genes from the gene pool and query samples were respectively calculated. If the BCR
values from the reference and query samples were lower than the threshold value, it was considered that the gene from the reference genome did not share homology with the query gene, and the gene from the query genome was added to the gene pool. All the query samples were analysed using this method, yielding the final pan gene pool.

Metabonomics analysis of SEM-2 fermentation broth

The strain SEM-2 was inoculated into NB medium at a volume ratio of 1:99 and incubated for 48 h at 37°C and 200 rpm. Subsequently, the SEM-2 fermentation broth was centrifuged to isolate the supernatant. Blank NB medium without SEM-2 inoculation was used as the control. Six biological replicates of both the fermentation broth and blank control were employed. The main metabolites in the fermentation broth of strain SEM-2 were detected and analysed by liquid chromatography-quadrupole-time of flight-mass spectrometry (LC-QTOF-MS) (Chen et al. 2013).

Statistical analysis

SPSS statistics software was used to analyse the significance of difference of the data obtained, and multiple pairwise comparisons were conducted using Tukey’s test.

Results

Isolation and identification of strain SEM-2

Strain SEM-2 was isolated from silkworm excrement by plating on inorganic phosphorous selective medium (using calcium phosphate as the sole phosphorus source). The strain grew well on this medium, and colonies were white, opaque, round and folded (Fig. 1A). Furthermore, Gram staining was positive.
(Fig. 1B). The strain has been deposited at the Guangdong Culture Collection Center under Accession No. GDMCC 60039.

**Genome sequence and bioinformatics analyses of strain SEM-2**

Using the Illumina HiSeq 4000 sequencing platform, 646 Mb of data were acquired for sample SEM-2A. Based on the assembly results, the genome size was 4,083,029 bp, the GC content was 43.64%, the number of scaffolds was 20 (with an N50 length of 2,129,282 bp), and the number of contigs was 34 (with an N50 length of 625,361 bp). A total of 4246 genes were predicted based on the genome sequence of strain SEM-2, and genes were searched against the COG databases using BLAST. A total of 3209 genes were classified into 25 COG categories (Fig. 2), along with 72 tRNAs and five rRNAs (Table 1). BLAST analysis of the genome sequence revealed the highest sequence similarity with *B. subtilis*. The results of core- and pan-genomic analyses between strain SEM-2 and *B. subtilis* 168 (NC_000964.3) (Kunst et al. 1997) showed that the number of core, pan and dispensable genes was 3945, 4606 and 661, respectively. Thus, the SEM-2 genome appears to have some specific sequence features. The genome sequence of *B. subtilis* SEM-2 has been deposited in GenBank under Accession No. NGUK00000000.

**Antagonism of strain SEM-2 against Fusarium spp.**

Antagonism and morphological analyses were conducted using the flat confrontation method and scanning electron microscopy (SEM), respectively. The results showed that strain SEM-2 clearly inhibited the mycelial growth of all the four *Fusarium* spp. examined (Fig. 3A). Furthermore, SEM observations showed that the hyphae of *F. graminearum* in the co-culture were seriously damaged, with surface shrinkage and leakage of cell contents, when compared with the control (Fig. 3B). In addition,
the results of conidia germination tests revealed that the SEM-2 culture filtrate significantly inhibited the germination of *F. graminearum* spores (Fig. 3C). The inhibitory effect became progressively obvious with the increasing concentration of the filtrate; however, the conidia germination rate began to increase with the treatment time and the inhibitory effect weakened (Fig. 3C).

**Effect of strain SEM-2 on disease occurrence in tomato seedlings**

The inhibitory effect of strain SEM-2 on crop diseases was analysed on *Fusarium*-infected tomato seedlings using the pot experiment. The results showed that tomato seedlings grew well for 1–3 weeks under normal soil conditions. However, in soil mixed with a low concentration of *Fusarium* spores, disease symptoms emerged in the blank group in the second week. Among the groups treated with different concentrations of SEM-2-G, only the group treated with 10^6 CFU/mL SEM-2-G exhibited 10% disease incidence, while the other two groups grew well in the second week without disease. However, in the third week (21 days), disease symptoms were noted in the blank group and the three groups treated with different concentrations of SEM-2-G. The blank group without added bacterial suspension exhibited a 46.7% disease incidence, which was significantly higher than those of the other three groups treated with different concentrations of SEM-2-G. Among the groups treated with high concentrations of *Fusarium* spores, the blank group presented disease symptoms in the first week (7 days), and the disease incidence was 16.7% and 43.3% in the second and third weeks, respectively, which were significantly higher than those observed for the other bacterial suspension treatment groups during the same period (Fig. 4 I and II).

By contrast, the root system development of tomato seedlings of all the groups was observed after 21 days. The root system development of tomato seedlings was significantly different among the normal
control group, low spore concentration group (without SEM-2-G addition), high spore concentration
group (without SEM-2-G addition) and groups treated with SEM-2-G suspension (Fig. 4 III). The
fibrous roots of tomato seedlings in soil with different concentrations of *Fusarium* spores were relatively
scarce, while the root system was developed and the fibrous roots were abundant in the normal control
group and groups treated with SEM-2-G suspension.

*Analysis of putative secondary metabolites of strain SEM-2*

The putative secondary metabolites biosynthetic gene clusters of strain SEM-2 were identified and
categorised using antiSMASH 4.0 (https://antismash.secondarymetabolites.org/#/start). Genes involved
in the biosynthesis of the antimicrobial compounds bacillaene, fengycin, bacillibactin, subtilosin A,
surfactin and bacilysin were identified in the genomic sequence of strain SEM-2 (Table 2). Analysis of
the fermentation supernatant of strain SEM-2 by LC-QTOF-MS determined 1285 differentially
expressed substances that were not detected in the blank control. KEGG analysis of these differentially
expressed substances identified 50 related KEGG pathways, among which metabolic pathways,
synthesis of secondary metabolites and synthesis of antibiotic pathways were well represented (Fig. 5).

*Colonisation of tomato rhizosphere by strain SEM-2-G*

Fluorescence microscopy results showed that many GFP-labelled SEM-2-G cells retained the ability to
grow in the roots of tomato plants after being fully submerged in sterile water (Fig. 6B, C).
Subsequently, the roots were surface-cleaned and disinfected, ground thoroughly, suspended in sterile
water and plated on NB agar containing 10 μg/mL chloramphenicol. Following 48 h of incubation, a
large number of green fluorescent colonies were noted (Fig. 6D). Similar results were also observed on
the roots of the tomato plants and rhizosphere soil in the pot experiment. These findings implied that
strain SEM-2-G could colonise the roots of tomato plants, which may play an important role in the
successful control of the plant disease.

Discussion

The strain SEM-2 was isolated during the high-temperature stage of silkworm excrement composting
(fermentation temperature, 60–65°C). BLAST analysis revealed highest sequence similarity with B. subtilis. The genome of strain SEM-2 was found to comprise 4,083,029 bp with 43.64% GC content. When compared with the genome size of B. subtilis SEM-9 (genome size, 4,121,982 bp; GC content, 43.80%), that of strain SEM-2 presented slight differences (Li et al. 2019). For example, the annotated genes involved in the biosynthesis of the antimicrobial compounds in the genome of strain SEM-2 mainly included bacillaene, fengycin, bacillibactins, subtilosin A, surfactin and bacilysin; however, only fengycin, subtilosin A, surfactin and bacilysin were detected in strain SEM-9. In addition, the results of core- and pan-genomic analyses between strain SEM-2 and B. subtilis 168 (NC_000964.3) (Kunst et al. 1997) revealed 3945 core genes, 4606 pan genes and 661 dispensable genes. Thus, the SEM-2 genome appears to have some specific sequence features.

Strain SEM-2 presented a marked function of inhibiting the growth of Fusarium spp., and showed a significant effect on controlling Fusarium disease in the pot experiment. The inhibitory effect of strain SEM-2 on the germination rate of Fusarium spores was noted to be positively related to the concentration of the fermentation broth. However, the conidia germination rate increased with the treatment duration and the inhibitory effect of the bacterial strain weakened. Besides, in the low spore concentration group in the pot experiment, the disease onset time in the sub-group treated with 10^6
CFU/mL SEM-2-G suspension (LB) was earlier than that in the sub-groups treated with $10^7$ and $10^8$
CFU/mL SEM-2-G suspension (LC and LD, respectively). In contrast, in the high spore concentration
 group, there was an increase and no significant difference in the disease incidence in the third week
among the three sub-groups treated with different concentrations of SEM-2-G. The disease incidence in
the sub-groups treated with $10^5$ and $10^7$ CFU/mL SEM-2-G (LA and HA, respectively) in the third week
was close to 50%; however, the disease incidence among the low spore concentration groups (LB, LC
and LD) was significantly lower than that among the high spore concentration groups (HB, HC and
HD). Based on the results of spores germination and the pot experiment, it can be speculated that the
antagonistic effect of strain SEM-2 on *Fusarium* spp. is very significant in the early stage of the disease
or under low pathogen concentrations, and becomes weaker and disease control becomes difficult with
the increase in pathogen concentration or disease severity. Therefore, strain SEM-2 may have a better
control effect during the early stage of disease occurrence, and exhibit only a slight effect during the
middle and late stages of the disease. Hence, comprehensive control measures may be necessary for the
control of diseases caused by *Fusarium* spp. (Raza et al. 2016).

Furthermore, strain SEM-2 displayed potent antagonistic activity against *Fusarium* spp., without
the need for close contact in the plate confrontation experiment. We therefore speculated that strain
SEM-2 may inhibit or antagonise the pathogenic *Fusarium* spp. by secreting antimicrobial substances.
To analyse the possible antagonistic substances secreted by strain SEM-2 against *Fusarium* spp., gene
sequence and non-targeted metabonomic analyses were performed. The genes involved in the
biosynthesis of the antimicrobial compounds, bacillaene, fengycin, bacillibactins, subtilosin A, surfactin
and bacilysin, were identified in the genome of strain SEM-2, and analysis of non-targeted metabolites
revealed significant changes in pathways related to antibiotic synthesis. Most of these antibiotics were
noted to be polyene and ester peptide antibiotics, with different antimicrobial mechanisms. Bacillaene is a polyene antibiotic that inhibits the synthesis of prokaryotic proteins, but not eukaryotic proteins (Patel et al. 1995). Therefore, bacillaene may not be related to the *Fusarium*-antagonising function of strain SEM-2, but may be associated with the inhibition of other bacterial pathogens (Harwood et al. 2018).

Fengycin, bacillibactin, subtilosin A and surfactin are lipopeptide antibiotics, among which surfactin and fengycin exert antifungal activities (Ahimou et al. 2000; Babasaki et al. 1985; Peypoux et al. 1999; Singh and Cameotra 2004; Yoneda et al. 2001). Fengycin is a potent inhibitor of filamentous fungi, which may function by making the plasma membrane of target cells more permeable (Deleu et al. 2005, 2008; Patel et al. 2011; Vanittanakom et al. 1986). Therefore, we speculate that fengycin and surfactin may be the major antibiotics of strain SEM-2, which inhibit and damage the hyphal growth of *Fusarium* spp. In a previous study, Song et al. demonstrated that bacterial treatment twisted and shrivelled the pathogen hyphae, which may be an indicator of direct damage caused by antifungal substances (Song et al. 2014).

In addition to secreting antibacterial substances, successful colonisation in the roots of plants is also one of the important factors for biocontrol strains to prevent soil-borne diseases (Shafi et al., 2017), as well as the premise to further compete for nutrition and living space with the pathogen and induce a stress response in plants. In a previous study, colonisation assays demonstrated that *B. subtilis* 9407 could extensively colonise melon roots and leaves, and that the biocontrol activity of *B. subtilis* 9407 is the result of coordinated action of surfactin-mediated antibacterial activity and colonisation (Fan et al. 2017). In the present study, strain SEM-2-G successfully colonised in the roots and rhizosphere soil of tomato plants, which may be one of the direct factors controlling Fusarium disease in tomato.

**Conclusion**
B. subtilis SEM-2 was isolated from silkworm excrement and its antagonistic effect on Fusarium spp., genome sequence and secondary metabolites were studied. Strain SEM-2 displayed good antagonistic and disease control activity against Fusarium spp., secreted antibacterial substances and colonised the roots of tomato seedlings (probably via the surface of roots), which may be important factors for its biocontrol function. Thus, B. subtilis SEM-2 has significant potential as a biocontrol agent against Fusarium wilt.

Acknowledgements

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**Figure and Table Legends**

**Fig. 1. Isolation (A) and Gram staining (B) of strain SEM-2**

**Fig. 2. Circular maps of strain SEM-2 genome sequence**

From outer to inner, the 1st circle shows the ncRNA result of the positive strand, containing tRNA, rRNA and sRNA; the 2nd circle reveals the COG function of the positive strand along the chromosome, with each colour representing a function classification; the 3rd circle indicates the ncRNA result of the negative strand; the 4th circle denotes the COG function of the negative strand; the 5th circle shows the GC content (black); and the 6th circle presents the GC skew ((G-C)/(G+C), green > 0, purple < 0). The 5th and 6th circles are plotted relative to the average value.

**Fig. 3. Antagonistic effect of strain SEM-2 against *Fusarium* spp.**

(A) Antagonistic effect of strain SEM-2 against four *Fusarium* spp. determined using the flat confrontation method. a–d: *Fusarium catenulatum, F. oxysporum, F. solani* and *F. graminearum*; SEM-2, *Fusarium* spp. co-cultured with strain SEM-2; SEM-3, *Fusarium* spp. co-cultured with strain SEM-3; CK, *Fusarium* spp.; (B) Mycelial growth of *F. graminearum* in the presence of strain SEM-2 in confrontation cultures analysed by SEM. SEM-2, Mycelia of *F. graminearum* co-cultured with strain SEM-2; CK, Mycelia of *F. graminearum* cultured without strain SEM-2; (C) Effect of strain SEM-2 on conidia germination rate of *F. graminearum*. CK, Mixed suspension of *F. graminearum* conidia and sterile water at a ratio of 1:1. Values (12.5%, 25% and 50%) indicate different concentrations of cell-free SEM-2 culture filtrate (0.1 g freeze-dried powder/mL sterile water) used. Bars represent the mean ± standard error value (STDEV). Within a time point, two samples with the same letter are not significantly different ($p < 0.01$).
Fig. 4. Effect of strain SEM-2 on disease occurrence in tomato seedlings

Note: CK, Normal control group without *Fusarium* spores and strain SEM-2; L, Low spore concentration group (*Fusarium* spore suspension at a concentration of $10^5$ CFU/mL was mixed with soil); H, High spore concentration group (*Fusarium* spore suspension at a concentration of $10^7$ CFU/mL was mixed with soil); A, the group was treated with 10 mL of blank medium lacking strain SEM-2-G; B, C and D, groups were treated with 10 mL of SEM-2-G suspension at a concentration of $10^6$, $10^7$ and $10^8$ CFU/mL, respectively. I and II depict the effect of strain SEM-2 on tomato seedling disease at 14 and 21 days, respectively; III shows tomato seedling root system development after 21 days. CK is the normal control group without *Fusarium* spores and strain SEM-2; LA is the low spore concentration group without strain SEM-2-G; HA is the high spore concentration group without strain SEM-2-G; LD is the low spore concentration group with 10 mL of SEM-2-G suspension at a concentration of $10^8$ CFU/mL; HD is the high spore concentration group with 10 mL of SEM-2-G suspension at a concentration of $10^8$ CFU/mL. SPSS statistical software was used to analyse the significance of differences between different groups, and multiple pairwise comparisons were made using Tukey’s tests. Lower case letters indicate significant differences at $p < 0.05$.

Fig. 5. KEGG analysis of metabolites in the fermentation supernatant of strain SEM-2 assayed by LC-QTOF-MS

Differentially abundant substances in the fermentation supernatant of strain SEM-2 compared with blank controls determined by LC-QTOF-MS. For biological duplicates, replicates were combined and averaged, and $p$-values from Student’s t-tests and VIP values from orthogonal projections to latent
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**Fig. 6. Fluorescence microscopic analysis of colonisation of tomato roots by strain SEM-2-G**

(A) Tomato roots without strain SEM-2-G (Control); (B and C) Tomato roots treated with SEM-2-G at different magnifications; (D) Colonies of strain SEM-2-G on NA medium containing ground SEM-2-G-colonised tomato root pulp.
### Table 1 Genome features of *B. subtilis* SEM-2

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Gene Internal Length</th>
<th>Gene Internal GC Content</th>
<th>Gene Number</th>
<th>Gene Length</th>
<th>%GC Content</th>
<th>% of Genome (Genes)</th>
<th>Gene Average Length</th>
<th>% of Genome (internal)</th>
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<td>11.82</td>
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</table>
Table 2 Analysis of putative secondary metabolites biosynthetic gene clusters using antiSMASH 4.0

<table>
<thead>
<tr>
<th>Cluster No.</th>
<th>Gene cluster type</th>
<th>Notes</th>
<th>Base start No.</th>
<th>Base end No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>clu7</td>
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</tbody>
</table>

Note: Secondary metabolite types detected by AntiSMASH; transatPKS, transatPKS gene cluster; NRPS, Nonribosomal peptide synthetase gene cluster; Sactipeptide, Sactipeptide gene cluster; Saccharide, Saccharide peptide gene cluster. Other gene clusters contain a secondary metabolite-related protein that does not fit into any other category. According to the AntiSMASH results, homologous genes were selected based on a BLAST E-value <1E-05, ≥30% sequence identity, and a shortest BLAST alignment coverage >25%.
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Note: CK, Normal control group without Fusarium spores and strain SEM-2; L, Low spore concentration group (Fusarium spore suspension at a concentration of 105 CFU/mL was mixed with soil); H, High spore concentration group (Fusarium spore suspension at a concentration of 107 CFU/mL was mixed with soil); A, the group was treated with 10 mL of blank medium lacking strain SEM-2-G; B, C and D, groups were treated with 10 mL of SEM-2-G suspension at a concentration of 106, 107 and 108 CFU/mL, respectively.

I and II depict the effect of strain SEM-2 on tomato seedling disease at 14 and 21 days, respectively; III shows tomato seedling root system development after 21 days. CK is the normal control group without Fusarium spores and strain SEM-2; LA is the low spore concentration group without strain SEM-2-G; HA is the high spore concentration group without strain SEM-2-G; LD is the low spore concentration group with 10 mL of SEM-2-G suspension at a concentration of 108 CFU/mL; HD is the high spore concentration group with 10 mL of SEM-2-G suspension at a concentration of 108 CFU/mL. SPSS statistical software was used to analyse the significance of differences between different groups, and multiple pairwise comparisons were made using Tukey’s tests. Lower case letters indicate significant differences at p < 0.05.
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