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Identification and biocontrol potential of antagonistic bacteria strains against *Sclerotinia sclerotiorum* and their growth-promoting effects on *Brassica napus*

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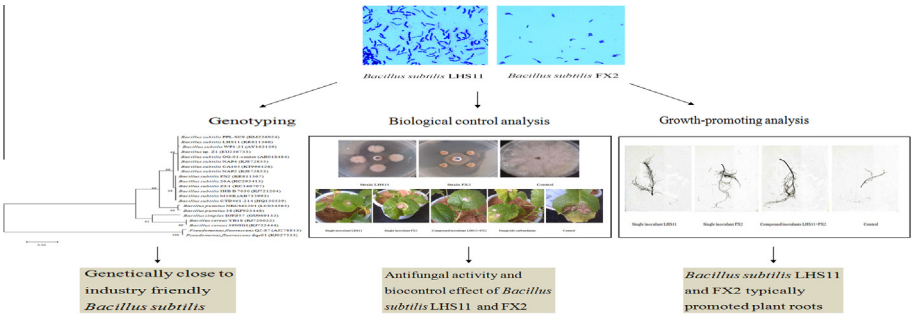
HIGHLIGHTS

- 19 PGPR had the capacity of phosphate solubilizing, nitrogenase and IAA secretion.
- *Bacillus subtilis* LHS11 and FX2 showed antifungal activity.
- Inoculant (LHS11 + FX2) had biocontrol and growth-promoting capacity in rapeseed.

GRAPHICAL ABSTRACT



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ABSTRACT

In order to assess the potential of plant growth promoting rhizobacteria (PGPR) to protect rapeseed plants against *Sclerotinia sclerotiorum* (Lib.) de Bary, antifungal properties and growth-promoting effects of PGPR were evaluated. Phosphate solubilization, nitrogenase and IAA secretion of 19 strains were tested. Out of 19 strains, 13 could solubilize phosphate (3.79–204.74 mg/L), 10 strains produced IAA (4.34–54.36 mg/L) and 14 strains had nitrogenase activity (7.14–246.46 nmol/mL·h). All strains were tested for their antagonism against *S. sclerotiorum in vitro* based on panel confrontation method. Strain LHS11 efficiently antagonized *S. sclerotiorum* and its inhibition rate reached 85.71%. In greenhouse experiments, the control efficiency of compound inoculant (LHS11 + FX2) reached 80.51%. The compound inoculant significantly increased the plant height (217.76 mm), shoot fresh weight (1.7794 g), root fresh weight (0.0495 g) and root dry weight (0.0086 g). Based on 16 S rDNA sequence alignment and several biochemical and physiological characteristics, strains LHS11 and FX2 were identified as *Bacillus subtilis*. Therefore, these results strongly suggested that *B. subtilis* LHS11 and FX2 are promising biocontrol and growth-promoting agents in rapeseed plants.

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1. Introduction

Rapeseed (*Brassica napus* L.) is an important oil crop in many parts of the world. In China, the annual planting area is >7.2 million hectares and its total seed yield can reach up to 13.5 million tons (Yin et al., 2009). Due to the changes in temperature, rainfall and other climatic conditions, various plant diseases have been occurring on rapeseed plants in recent years. *Sclerotinia* stem rot (SSR) caused by *Sclerotinia sclerotiorum* is particularly serious. The disease is widespread wherever rapeseed is cultivated in China. Especially, the incidence of SSR might reach 80% in the regions along

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the Yangtze River basin. SSR can reduce not only the yield of rapeseed (from 10% to 80%) but also oil quality (Gao et al., 2014).

Sclerotinia sclerotiorum primarily spreads by spores and usually in forms of sclerotia, which could infect stems, leaves, flowers and siliques, even spread easily to adjacent plants (Zhou and Boland, 1998). Sclerotia of *S. sclerotiorum* could reside in the soil for several years and, when appropriate environmental conditions exist, germinate either in a myceliogenic manner, giving rise to infective hyphae, or by carpogenic germination to produce apothecia which release millions of sexually produced, air-borne ascospores (Bardin and Huang, 2001; Coley-Smith and Cooke, 1971). At present, the application of chemical synthetic pesticides is much more effective than cultivation management, but chemical synthetic pesticides have negative environmental impact and their efficacy can decrease as time goes by (Wang et al., 2015). Compared to chemical control, using micro-organisms could be an environmentally-friendly component of an integrated management program to control plant diseases, especially using plant growth promoting rhizobacteria (PGPR) (Rahman et al., 2016; Kamal et al., 2016).

Several strains of PGPR (*Bacillus* spp., *Pseudomonas* spp.) have received much attention previously in the prevention of *S. sclerotiorum* (Simonetti et al., 2012). Chen et al. (2014) reported that good biocontrol efficacy against *S. sclerotiorum* on rapeseed was achieved by spraying cell suspension of *B. subtilis* strain EDR4, and scanning electron microscopy revealed that EDR4 cells significantly suppressed the hyphal growth of *S. sclerotiorum*. In addition, *Clonostachys rosea*, *Trichoderma harzianum*, *T. hamatum*, *Alternaria atra*, *Paraphaeosphaeria minitans*, etc. were also reported to inhibit the growth of *S. sclerotiorum* (Rodríguez et al., 2015; Zhang et al., 2016; Huang and Erickson, 2008; Jones et al., 2014). In general, competition for nutrients, niche exclusion, induced systemic resistance and antifungal metabolite production are the chief modes of biocontrol activity in PGPR (Lugtenberg and Kamilova, 2009). Some bacteria produce a wide spectrum of antibiotics as secondary metabolites, like phenazine, lipopeptide, 2, 4-diacetylphloroglucinol, pyoluteorin, benzothiostrobin, etc. (Xu et al., 2015; Jain et al., 2015; Selin et al., 2010; Alvarez et al., 2012; Berry et al., 2010; Défago, 1993; Maurhofer et al., 1994).

In addition, several PGPR strains may mainly promote plant growth by increasing nutrient availability, promoting absorption of nutrients, improving the nutritional status and helping plants to adapt to a number of environmental stresses, etc. Among the processes that contribute to increasing nutrient availability to plant roots, phosphorus solubilization (Kumar et al., 2014), IAA production (Glickmann and Dessaux, 1995) and nitrogen fixation (Malik et al., 1997) are the recognized mechanisms of plant growth promotion due to the importance of limiting factors for crop productivity. In recent years, several bacterial species are often associated with the plant growth, yield and crop quality, such as *Bacillus* and *Pseudomonas* (Ahmed et al., 2014; Aeron et al., 2011; Orhan et al., 2006). The root is an important organ to absorb nutrients and water, affecting the growth of crops and the absorption of nutrients by morphological development. Root characteristics (total root length, root surface area, root diameter and root volume, etc.) play a decisive role on nutrient availability (Kapulnik et al., 1985).

The objectives of this study were to find efficient PGPR strains that can be used as plant growth promoting and biocontrol agents by (a) elucidating growth promoting properties of 19 PGPR strains, (b) evaluating inhibiting ability of these PGPR strains *in vitro*, (c) investigating the potential of microbial inoculants for practical antifungal application in greenhouse trials, (d) identifying, based on genetic and phenotypic characteristics, potential application strains, (e) measuring the plant growth promoting ability of microbial inoculants in pot experiments.

2. Materials and methods

2.1. Strains isolation and storage

PGPR (strains JM170, JM92, LX191, G, JX59, LX22, LX81, LHS11, LM4-3, 4N4, P2-1, PGRS-3, XX1, XX2, XX5, XX6, FX1, FX2 and F1-4) were isolated from the rhizosphere of various plants, such as wheat (*Triticum aestivum*), corn (*Zea mays*), alfalfa (*Medicago sativa*) and clover (*Trifolium pratense*) (Table 1), and were procured from the culture collection of the Key Laboratory of Grassland Ecosystem, College of Grassland Science, Gansu Agricultural University, Lanzhou, Gansu, China. LB agar medium was used for the bacterial growth and storage (Sambrook and Russel, 2001), and liquid LB medium was used for testing antagonistic activity.

The fungal pathogen *S. sclerotiorum* was kindly provided by College of Grassland Science, Gansu Agricultural University, Gansu province, China, cultured on potato dextrose agar (PDA) medium.

Pikovskaya's agar medium (PKO) was used for separating phosphate-dissolving strains (Pikovskaya, 1948), and liquid Pikovskaya's medium was used for the quantitative estimation of phosphate solubilization; Liquid King's B medium was used for the determination of indole-3-acetic acid (IAA) (Glickmann and Dessaux, 1995); Nitrogen-free medium (NFM) was employed for detecting nitrogenase activity (Hafeez and Malik, 2000).

Solid carriers such as peat, charcoal and flower soil (2:2:1, w/w) were taken as supporting materials for the growth of biocontrol bacteria. Equal amounts of each solid carrier was mixed with distilled water and stirred thoroughly to form a slurry or paste (Page et al., 1982).

2.2. Growth promoting properties of bacterial strains

2.2.1. Qualitative and quantitative estimation of phosphate solubilization

Each rhizobacterial isolate was spot inoculated on Pikovskaya's agar plate amended with bromophenol blue to test phosphate solubilization ability (Subba Rao, 1982). The formation of phosphate solubilization zone was observed (dividing phosphate solubilization zone on Pikovskaya's agar by growth diameter of spot inoculant) after 5 days of incubation at 28 °C. The method developed by Pikovskaya (1948) was used for quantitative estimation of tricalcium phosphate solubilization by the isolate in the liquid Pikovskaya's medium. One mL culture supernatant was made to form final volume of 5.0 mL with distilled water and 5.0 mL ammonium molybdate was added. The mixture was thoroughly shaken. The contents of the flasks were diluted to 20 mL. One mL chlorostannous acid was added and diluted with distilled water to 25 mL in a volumetric flask. The contents were mixed thoroughly and the blue colored intensity was measured after 10 min at 660 nm and the amount of phosphate released was determined using the calibration curve of KH_2PO_4. An appropriate blank was

Table 1
The source of nineteen plant growth promoting rhizobacteria strains for testing.

Strains	Host plant	Strains	Host plant
Bacillus sp. JM170	*Medicago sativa*	P2-1[a]	*Zea mays*
Pseudomonas sp. JM92	*Medicago sativa*	PGRS-3[a]	*Poa alpigena*
Azotobacter sp. LX191	*Triticum aestivum*	XX1[a]	*Medicago sativa*
Azospirillum brasilense G	*Triticum aestivum*	XX2[a]	*Medicago sativa*
Bacillus sp. JX59	*Triticum aestivum*	XX5[a]	*Medicago sativa*
Bacillus sp. LX22	*Triticum aestivum*	XX6[a]	*Medicago sativa*
Bacillus sp. LX81	*Triticum aestivum*	FX1 [a]	*Medicago sativa*
LHS11[a]	*Trifolium pratense*	FX2[a]	*Medicago sativa*
LM4-3[a]	*Medicago sativa*	F1-4[a]	*Medicago sativa*
4N4[a]	*Zea mays*		

[a] No identification.

kept in which all reagents were added except the culture (Sundara and Sinha, 1963).

P. solubilizaton = T-C

where T = PVK with TCP, inoculated; C = PVK with TCP, un-inoculated

2.2.2. Qualitative and quantitative estimation of indole-3-acetic acid (IAA) production

The indole-3-acetic acid (IAA) production was detected as described by Brick et al. (1991). Bacterial cultures were grown for 48 h in liquid King's B medium at 36 ± 2 °C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 mL) was mixed with two drops of orthophosphoric acid and 4 mL of the Salkowski reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 M $FeCl_3$ solution). Development of pink colour indicates IAA production. Quantitative measurement was done by colorimetric method (Glick, 1995) with slight modification. 2–3 drops of orthophosphoric acid were added to 2 mL of supernatant and 4 mL of Salper reagent (2 mL of 0.5 M $FeCl_3$) in 98 mL of 35% $HClO_4$. This mixture was incubated at room temperature in dark for 25 min. Absorbance was measured at 535 nm for the development of pink color. Concentration of indole-3-acetic acid was estimated by preparing calibration curve using indole-3-acetic acid (IAA, Hi-media) as standard (10–100 mg/L).

2.2.3. Quantitative estimation of nitrogen fixation

The nitrogen-fixing ability of isolates was tested using the acetylene reduction assay (ARA), as described by Boddey and Knowles (1987). The nitrogenase activity was measured after growth in 10 mL vials containing 4 mL of semi-solid (0.18% agar-agar) nitrogen-free medium. After 24 h of incubation at 28 °C in the dark, the vials were sealed with rubber septa. Meanwhile, 10% (v/v) of the air phase was replaced with acetylene (Burris, 1972). After the cultures were incubated for 1 h in acetylene, the amount of C_2H_4 produced was measured for three vials for each isolate using a Clarus 600 gas chromatograph (Perkin Elmer) with a Col-Elite-Alumina column (50 m × 0.53 mm ID × 10 μm) and a flame ionization detector connected to a chromatography data computer system.

2.3. Antagonism against S. sclerotiorum

2.3.1. Antagonistic activity of PGPR against S. sclerotiorum in vitro

Antagonistic activity of the PGPR against *S. sclerotiorum* was evaluated on PDA plates by dual culture technique. Bacterial isolates were incubated in liquid LB medium at 25 °C. Fungal pathogen was grown on PDA medium. Five-day-old mycelial disc (5 mm) was placed in the centre of PDA medium. An exponentially growing bacterial culture (10^8 cfu/ml) was spotted 2 cm juxtaposed from the fungal disc. The plates were incubated at 28 ± 1 °C for 3–7 days. The percentage of growth inhibition (I) was calculated by measuring the distance between the edges of the bacterial and fungal colonies by using the formula:

$$I(\%) = [(C - T)/(C - C_0)] \times 100$$

where C is the radial growth of fungus in control and T is the same in dual culture (Aeron et al., 2011). C_0 means the diameter of the test fungus agar discs (5 mm).

In order to investigate the antagonistic effect on mycelial morphology, microscopic examination were made by optical microscope at a magnification of 40×. Mycelium growth was observed near antagonistic inhibition zone edge, and the normal mycelium of pathogenic fungus was regarded as control.

2.3.2. Antifungal activity of cell-free fermentation liquid

Antifungal activity of PGPR cell-free fermentation liquid was described by Wang et al. (2011). PDA was used as the medium for *S. sclerotiorum*. The 2% (V/V) bacterial suspension was inoculated to the liquid LB medium and incubated at 28 °C for 48 h at 180 rpm. The fermentation liquid was centrifuged at 8000 rpm for 10 min, and was filtered by 0.22 μm filtration membranes. The media incorporating the filtrate at a volume fraction of 10% were inoculated with agar discs containing the tested fungus (5 mm) in the centre. Three replicate plates for the fungus were incubated at 27 ± 2 °C. Control plates containing the medium mixed with sterile water (10%, by volume) were included. After incubation for 3–7 days, the diameter of mycelium growth of the fungus (mm) in both treated (T) and control (C) Petri dishes was measured in perpendicular directions until the fungus growth in the control dishes was almost complete. The percentage of growth inhibition (I) was calculated using the formula:

$$I(\%) = [(C - T)/(C - C_0)] \times 100.$$

C_0 means the diameter of the test fungus agar discs (5 mm).

2.4. Antagonistic determination between strains LHS11 and FX2

Oxford cup method was used to measure antagonistic reaction between strains LHS11 and FX2. The 2% (V/V) bacterial suspension of strain LHS11 was inoculated to the liquid LB medium at 28 °C for 48 h at 180 rpm. The fermentation liquid was centrifuged at 8000 rpm for 10 min, and was filtered by 0.22 μm filtration membranes. Then the 100 μL filtrate was dropped in Oxford cup (diameter 7 mm) in the centre of LB agar plate that contained 2% strain FX2, and sterile water was used as negative control. The plates were observed for inhibition zone after 24 h of incubation at 28 °C and experiment was replicated thrice.

2.5. Biocontrol on rapeseed leaves in vitro

Rapeseed seeds (*B. napus* L. Long You NO.9) were obtained from College of Grassland Science, Gansu Agricultural University, Gansu province, China. The seeds were surface sterilized with 0.1% potassium permanganate ($KMnO_4$) for 10 min and immediately washed with sterile distilled water 3–4 times. Ten seeds were planted in one culture pot (11 cm × 10 cm × 8 cm) filled with 250 g cultivation soil in the greenhouse (Li et al., 2011), and each treatment consisted of three potted plants.

Biocontrol effect of inoculant was carried out as described by Cheng et al. (2014). When the rapeseed had 7 leaves, 3 uniform size leaves per pot were collected and respectively cultured on water agar medium that covered the petri dish (r = 45 mm). The fermentation liquid of the strains that were incubated in liquid LB medium at 25 °C for 24 h were sprayed on the leaf surface. Each leaf was sprayed with 1 mL fermentation liquid (OD 1.5 at 540 nm). The control was sprayed with distilled water. Then the leaves were inoculated with *S. sclerotiorum* mycelium discs (0.5 cm diameter), and kept moist for 24 h. The size of lesions was measured on the 3rd day after inoculation. The experiments were replicated thrice. The following treatments were investigated: (A–C) inoculated with strains; (A) single inoculant LHS11; (B) single inoculant FX2; (C) compound inoculant (LHS11 + FX2), the fermentation liquid of strains LHS11 and FX2 were pre-mixed in the ratio of 1:1 before spraying; (D) fungicide carbendazim (50% Wettable powder, WP, Sichuan Guoguang Agrochemical Co., Ltd.) and (E) control.

2.6. In vivo challenge experiment in greenhouse condition

In similarity with biocontrol test on leaves *in vitro*, when the rapeseed had 7 leaves, 3 uniform size plants per pot were retained and others were removed. The fermentation liquid of the strains that were incubated in LB liquid medium at 25 °C for 24 h were sprayed to the leaf surface. Each potted plant was sprayed with 10 mL fermentation liquid (OD 1.5 at 540 nm). The control was sprayed with distilled water. The leaves were inoculated with *S. sclerotiorum* mycelium discs (0.5 cm diameter), and kept moist for 24 h. The size of lesions was measured on the 3rd day after inoculation. The experiments were replicated thrice. The following treatments were investigated: (A–C) inoculated with strains; (A) single inoculant LHS11; (B) single inoculant FX2; (C) compound inoculant (LHS11 + FX2), the fermentation liquid of strains LHS11 and FX2 were pre-mixed within the ratio of 1:1 before spraying; (D) fungicide carbendazim at 0.05 g per pot and (*E*) control.

2.7. Identification of strains LHS11 and FX2

The identification of strains LHS11 and FX2 based on morphology, Gram staining, physiological and biochemical tests according to Bergey's Manual of Determinative Bacteriology (8th Chinese edition) (Buchanan and Bergey, 1984). Biochemical tests were performed by using standard protocol (Capsule, Motile, Anaerobic growth, Mannitol utilization, Penicillin antifungal, Phage lysis, Bead test, 5 °C, 50 °C, 1 g/100 mL NaCl, 5 g/100 mL NaCl, 7 g/100 mL NaCl). Further, the bacterial identification was confirmed by the 16S rDNA sequence, which was amplified from the purified genomic DNA by polymerase chain reaction (PCR) (BIO-RAD, California, USA). The forward primer (5′-GTTGTGAACTCCTTT TTCTCC-3′) and the reverse primer (5′-CGCAGAAACACAGGA TAGCA-3′) were used (He et al., 2005). The DNA fragments were amplified under the following conditions: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 10 min. The amplified DNA fragments were sequenced (Shenggong, Shanghai, China). Clustal W was used for alignment with corresponding sequences of 16S rDNA from the database using Blast (Thompson et al., 1994; Altschul et al., 1997). Phylogenetic trees were constructed with MEGA 5.0 by using the Neighbor-joining method with 1000 bootstrap replications.

2.8. Growth-promoting test in greenhouse

Strains LHS11 and FX2 were grown in LB liquid medium at 25 °C for 24 h at 150 rpm (pH 7.2). Every broth culture of 10^8 cfu/mL was utilized for inoculant preparation. The solid carrier materials were ground separately and air dried before mixing and curing followed by double sterilization (121 °C for 20 min). The sterile carrier materials (40 g each) were packed in recommended (50–70 μm thick) low density polythene bags of flexible sheets to protect from loss of moisture. The bags were sealed leaving about 25% airspace to give proper aeration to the inoculants. The strains (inocula) were mixed thoroughly with supporting carrier material under aseptic condition, and sealed and stored at room temperature. The initial count in each carrier-based preparation was made so as to obtain 10^8 cells/g at the time of storage (Aeron et al., 2011).

A short term pot-trial assay was carried out by placing sterile soil (250 g each) in earthen pots (11 cm × 10 cm × 8 cm). Pots were arranged in complete randomized block design with 5 replications. Pots were kept on a polyhouse bench and watered when required. Seeds with uniform shape and size were surface sterilized with 95% ethanol for 30 s, and then washed with sterile distilled water (5–6 times). The seeds were dried overnight under sterile air stream. Seed bacterization was done as described by Bhatia et al. (2008). Seeds coated with the strains and non-coated seeds were sown in pots in the following four sets of treatments. A: soil inoculated with rapeseed seeds bacterized with single inoculant LHS11; B: soil inoculated with rapeseed seeds bacterized with single inoculant FX2; C: soil inoculated with rapeseed seeds bacterized with compound inoculant (LHS11 + FX2); D: soil inoculated with rapeseed seeds (non-bacterized).

3 plants from each plot were randomly selected for recording on the 30th day after sowing and plant height, aboveground fresh weight, aboveground dry weight, underground fresh weight and dry weight were measured by conventional method. Meanwhile, total root length, root surface area, average root diameter, root volume and root length under each selected diameter class (0–0.5, 0.5–1.0, 1.0–1.5 and > 1.5 mm) were measured by using root scanner (LA2400 Scanner, Epson Expression 1000 XL). The resulting image was cropped to obtain a normalised image size (19 cm × 50 cm) in all treatments. Then, the images were analyzed with WinRHIZO Tron MF software (Regent, Quebec, Canada).

2.9. Statistical analyses

The data were statistically analyzed by using analysis of variance (ANOVA) for individual parameters on the basis of mean values to find out the significance at 5% level. The standard error of the mean, variance, and ANOVA statistics were calculated using SPSS software, version 17.0.

3. Results

3.1. Growth-promoting properties of strains

The substantial P-solubilization, IAA and nitrogenase activity by strains LHS11, LX191, LM4-3, JM170, JM92, 4N4, P2-1 and FX2 have clearly showed their inherent plant growth-promoting potential. Thirteen strains out of total 19 strains showed zone of phosphate solubilization on Pikovskaya's agar plate amended with bromophenol blue. Quantitative analysis of phosphate solubilization demonstrated that 9 strains (JM170, JM92, LX191, JX59, LX22, LX81, LHS11, 4N4 and P2-1) out of total 13 phosphate solubilizers showed phosphate solubilization higher than 100 mg/L (Table 2). Ten strains had the capability of producing IAA that was at

Table 2
Growth promoting properties of nineteen PGPR strains.

Strains No.	P-solubilization capacity (mg/L)	IAA secretion (mg/L)	Nitrogenase activity (C_2H_4 nmol/mL·h)
JM170	123.20 ± 3.65^{g}	4.34 ± 0.03^{g}	43.80 ± 2.11^{j}
JM92	132.60 ± 4.22^{f}	47.25 ± 1.67^{b}	75.34 ± 0.33^{h}
LX191	200.02 ± 2.15^{b}	54.36 ± 1.67^{a}	–
G	–*	–	180.90 ± 1.00^{c}
JX59	109.30 ± 0.55^{h}	–	–
LX22	152.69 ± 2.11^{e}	–	–
LX81	105.60 ± 0.13^{i}	–	102.34 ± 1.02^{f}
LHS11	204.74 ± 1.33^{a}	29.56 ± 0.13^{d}	220.36 ± 2.09^{b}
LM4-3	–	13.91 ± 1.67^{h}	246.46 ± 4.23^{a}
4N4	178.25 ± 3.15^{d}	17.80 ± 1.67^{e}	178.94 ± 0.03^{d}
P2-1	193.67 ± 2.14^{c}	16.84 ± 0.46^{f}	95.10 ± 2.56^{g}
PGRS-3	–	40.87 ± 1.56^{c}	–
XX1	35.70 ± 0.73^{k}	–	44.28 ± 0.00^{i}
XX2	11.80 ± 0.02^{l}	–	12.21 ± 0.00^{l}
XX5	–	10.01 ± 0.00^{i}	–
XX6	–	–	34.33 ± 1.33^{k}
FX1	–	–	7.14 ± 0.33^{n}
FX2	75.22 ± 2.09^{j}	15.30 ± 0.01^{g}	110.45 ± 0.04^{e}
F1-4	3.79 ± 0.01^{m}	–	10.06 ± 0.33^{m}

* No growth promoting properties. Values in the table are mean ± *SE*. Different superscript letters within the same column indicate significant difference at $P < 0.05$ level by Duncan's test.

4.34–54.36 mg/L level, and strain LX191 had the best capability. The nitrogenase activity of 14 strains ranged from 7.14 nmol/ mL·h to 246.46 nmol/ mL·h, and strain LM4-3 had the best effects. Strains LHS11, JM170, JM92, 4N4, P2-1 and FX2 had obvious P-solubilization capacity with both IAA secretion and nitrogen fixation ability.

3.2. Antagonism against S. sclerotiorum

3.2.1. Antifungal activity of PGPR strains in vitro

Four strains antagonized *S. sclerotiorum* efficiently, and the inhibitory rates were all >60% (Table 3, column A). After 3-day cultivation, strains LHS11 and FX2 had more significant antifungal activity ($P < 0.05$) than strains JM170 and LX22, and there was no significant difference between strains LHS11 and FX2. After 7-day cultivation, the control colony basically covered the petri dish (r = 45 mm). The antifungal activity of strain LHS11 reached 85.71% and was better than other strains. In addition, the antifungal activity of strain JM170 was weak, and the hyphae of *S. sclerotiorum* almost covered the whole dish but bypassed biocontrol agent.

Microscopic examination showed that strains LX22, JM170 and FX2 caused distortion and deformation to the mycelia of *S. sclerotiorum*. Strain LHS11 caused the release of mycelial protoplast on *S. sclerotiorum*, which resulted in the rupture of cell wall and the death of the pathogen. The control mycelia were uniform and smooth.

3.2.2. Antifungal activity of cell-free fermentation liquid with agar diffusion assay

The results indicated that cell-free fermentation broth from strains LHS11 and JM170 inhibit mycelial growth. After 3-day cultivation, the cell-free fermentation liquid of strains LHS11 and JM170 significantly inhibited the growth of *S. sclerotiorum*. Their inhibition rates were 60.55% and 46.79%, respectively (Table 3, column B). Similarly, the fermentation broth of the strains LHS11 and JM170 still showed clear antifungal activity on the 7th day, and their inhibition rates were 61.09% and 52.53%, respectively. The cell-free fermentation broth of strains FX2 and LX22 had no inhibitory effect.

3.3. Biocontrol on rapeseed leaves in vitro

We did not observe bacteriostatic ring after one day of the growth after disposing of Oxford cup method, and the bacteria grew well. It indicated that there was no antagonism between the strains LHS11 and FX2, in other words, they could coexist. *S. sclerotiorum* mycelium discs were inoculated after inoculation with the fermentation broth of different treatments on rapeseed leaves *in vitro*. Compared with the control (Table 4, column X), the biocontrol effect of compound inoculant (LHS11 + FX2) was more obvious and its control efficacy reached 83.75%. In addition, the control effect of treatments A, B and C were better than the treatment D with the fungicide carbendazim for SSR control ($P > 0.05$).

3.4. Control of the pathogen by inoculants in greenhouse challenge experiment in vivo

In greenhouse trials, the disease spots of treatment C were smaller than other treatments, and reached 3.67 mm. The biocontrol effect of compound inoculant (LHS11 + FX2) was higher than single inoculant and reached 80.51% (Table 4, column Y). The lesion diameter of treatment D rose up to 18.83 mm. The biocontrol efficacy of treatments A, B and C were better than the treatment D ($P > 0.05$).

3.5. Identification of strains LHS11 and FX2

3.5.1. Phenotypic, physiological and biochemical characteristics

The pure colonies of strain LHS11 on LB agar medium at 24th h after incubation were white, rough surface, smooth margin, opaque, drying and no pigment production. Similarly, the colonies of strain FX2 were milky white, convex surface, smooth margin, opaque, moist and no pigment production. The morphological characteristics of strains LHS11 and FX2 are as follows: the all cells were gram positive, motile rods, spore forming, non capsule, motile, and the spores were oval and subterminal. The physiological and biochemical test results are shown in Table 5. Strains LHS11 and FX2 were preliminarily identified as *B. subtilis* following Bergey's Manual of Determinative Bacteriology (8th Chinese edition) (Buchanan and Bergey, 1984).

3.5.2. Phylogenetic analysis by 16S rDNA analysis

Molecular characterization based on 16S rDNA homology of partial sequences (LHS11, 1467 bp; FX2, 1466 bp) with the sequences available in NCBI database website http://www. ncbi. nlm.nih.gov/blast (Altschul et al., 1997) confirmed the preliminary identification as *B. subtilis* (Bodour et al., 2003). Sequence was analyzed with corresponding sequences of different *Bacillus* isolates reported from different parts of the world. Strain LHS11 showed 99% homology with *B. subtilis* WP1-21 (AY162129, from USA) and *B. subtilis* PPL-SC9 (KM226924, from Korea). Similarly, strain FX2 showed 99% homology with *B. subtilis* 26A (KC295415, from China) and *B. subtilis* ZJ-1 (KC146707, from China). To trace the evolutionary patterns of the test isolates and to find the affinity with other selected sequences at NCBI, phylogenetic tree was also constructed using Neighbor-Joining method of mathematical averages (UPGMA) among 16S rDNA sequences of the strains and corresponding sequences of different *Bacillus* isolates. Phylogenetic tree (Fig. 1) verified LHS11 (KR811366) and FX2 (KR811367) as *B. subtilis* as the strains LHS11 and FX2 clustered closely with *B. subtilis*. To summarize, based on above morphological, biochemical, and molecular characterization, strains LHS11 and FX2 were identified as *B. subtilis*.

Table 3
Inhibition of the mycelial growth and antifungal activity of the selected strains metabolites against *S. sclerotiorum*.

Strain No.	3rd day		7th day	
	A[m]	B[n]	A	B
LHS11	83.75 ± 0.62^{b}	60.55 ± 1.33^{a}	85.71 ± 0.00^{a}	61.09 ± 1.67^{a}
FX2	88.13 ± 0.62^{a}	7.32 ± 0.33^{c}	82.14 ± 0.69^{b}	0.00 ± 0.00^{d}
JM170	59.41 ± 0.61^{d}	46.79 ± 0.67^{b}	67.46 ± 0.40^{d}	52.53 ± 0.6^{b}
LX22	66.25 ± 1.87^{c}	2.28 ± 0.33^{d}	75.40 ± 0.40^{c}	1.94 ± 0.33^{c}

[m] Antifungal activity of PGPR strains.
[n] Antifungal activity of cell-free fermentation liquid. Values in the table are mean ± *SE*. Different superscript letters within the same column indicate significant difference at $P < 0.05$ level by Duncan's test.

Table 4
Biocontrol efficacy of microbial inoculants against *S. sclerotiorum*.

Treatments	Lesion diameter (mm)		Biocontrol efficacy (%)	
	X[m]	Y[n]	X	Y
A	5.5 ± 1.04[d]	4.83 ± 0.60[c]	79.37 ± 3.90[b]	74.35 ± 3.19[b]
B	5.67 ± 0.33[c]	6.33 ± 0.67[b]	78.75 ± 1.25[c]	66.38 ± 3.55[c]
C	4.33 ± 0.67[e]	3.67 ± 0.72[d]	83.75 ± 2.50[a]	80.51 ± 3.86[a]
D	6.3 ± 0.33[b]	6.36 ± 0.33[b]	76.38 ± 2.22[d]	66.22 ± 3.46[d]
E	26.67 ± 0.67[a]	18.83 ± 0.83[a]	0.00 ± 0.00[e]	0.00 ± 0.00[e]

A Single inoculant LHS11.
B Single inoculant FX2.
C Compound inoculant (LHS11 + FX2).
D Fungicide carbendazim at 0.05 g per pot.
E Control (sterile water).
Values in the table are mean ± *SE*. Different superscript letters within the same column indicate significant difference at $P < 0.05$ level by Duncan's test.
[m] Biocontrol efficacy on rapeseed leaves *in vitro*.
[n] Biocontrol efficacy in potting experiments *in vivo*.

Table 5
Physiological and biochemical characters of strains LHS11 and FX2.

Characteristics	Strain LHS11	Strain FX2	*Bacillus. subtilis*
Gram character	+[a]	+	+
Capsule	–[b]	–	–
Motile	+	+	+
Anaerobic growth	–	–	–
Mannitol utilization	+	+	+
Glucose utilization	+	+	+
Gelatin hydrolysis	+	+	+
Tyrosine hydrolysis	–	–	–
Nitrate reduction	+	+	+
Penicillin antibacterial	–	–	–
Phage lysis	–	–	–
Bead test	–	–	–
5 °C	–	–	–
50 °C	–	–	–
1 g/100 mL NaCl	+	+	+
5 g/100 mL NaCl	D[c]	+	+
7 g/100 mL NaCl	D	D	+

[a] Positive for production.
[b] Negative for production.
[c] Variable for production.

3.6. Growth-promoting test in greenhouse

3.6.1. Effects of microbial inoculants on plant height and biomass of rapeseed

Compound inoculant (LHS11 + FX2) significantly affected the plant height and biomass of rapeseed. Compared with the control (Table 6, treatment D), single inoculant FX2 and compound inoculant (LHS11 + FX2) significantly increased plant height ($P < 0.05$), especially compound inoculant (LHS11 + FX2) (217.76 mm); Treatment C obviously increased the aboveground fresh weight (1.7794 g) ($P < 0.05$); Although the aboveground dry weight of different treatments also increased, there were no significant differences among the various treatments ($P < 0.05$); All microbial inoculants enhanced the underground fresh weight and dry weight of rapeseed. Treatment C markedly increased underground biomass ($P < 0.05$), and the underground fresh and dry weight respectively reached 0.0495 and 0.0086 g.

3.6.2. Effects of microbial inoculants on root morphology

Different microbial inoculants significantly affected the root morphology of rapeseed. The taproot and fibrous root of treatment D (control) were short and underdeveloped, and inoculation of different microbial inoculants resulted in developed roots, longer taproot, and more lateral roots. Compared with treatment D, the total root length (88.33 cm), the root surface area (10.98 cm^2) and the root diameter (0.377 mm) of treatment C were significantly higher (Table 7) ($P < 0.05$). The root volume (0.087 cm^3) of treatment B was greater than other treatments.

3.6.3. Effect of microbial inoculants on root length under each selected diameter class

Compared with the control (Table 8, treatment D), microbial inoculants significantly affected total root length with 0–0.5 mm root diameter. The treatment C resulted in longer roots than the other treatments, and reached 98.50 mm, while there were no significant difference between A, B and C ($P > 0.05$). Similarly, the total root length of roots with the diameter 0.5–1.0 mm was significantly higher after treatment C ($P < 0.05$), and reached 3.45 mm; Likewise, there were no significant difference between 1.0–1.5 mm and >1.5 mm ($P > 0.05$).

4. Discussion

In this study, the inhibitory rates of strains LHS11 and FX2 were all >80% by dual culture technique for inhibiting *S. sclerotiorum*. Previous studies revealed that the inhibitory rate of *B. subtilis* CKT1 reached 74.71% against *S. sclerotiorum in vitro* (Walia et al., 2013), while *Trichoderma harzianum* only 56.3% (Zhang et al., 2016). The cell-free culture filtrates of strains LHS11 and JM170 showed obvious antifungal activity to *S. sclerotiorum* on the 7th day after incubation, being similar with Zhang and Xue (2010). However, the cell-free culture filtrates of strains FX2 and LX22 did not show any inhibitory effect against *S. sclerotiorum*, and the bioactive metabolites may be produced upon induction-presence of the pathogen. The cell-free filtrate of strain LHS11 significantly suppressed mycelial growth *in vitro*. It suggests that inhibitory substances produced by strain LHS11 cells may play a major role in disease suppression (Jain et al., 2015; Monteiro et al., 2013). Strain FX2 causes the morphological alterations of *S. sclerotiorum* mycelia including increased branching, swelling and collapse of cytoplasm, related to competition and parasitism, and it is similar to the study of Rahman et al. (2016). In addition, our previous study showed that strains LHS11 and FX2 have broad antagonistic spectrum, as they significantly inhibited the mycelial growth of some pathogens, such as *Thanatephorus cucumeris*, *Fusarium oxysporum*, *Bipolaris sorokiniana*, *A. solani* and *F. oxysporum* f. sp. *cucumerinum* (Sun et al., 2014). This work has provided evidence that the mechanisms of strains LHS11 and FX2 are probably classified as competition, parasitism and antibiosis, and they can be good alternative biological resources for biocontrol of SSR.

The application of the bacterial strain on the rapeseed leaves 24 h before fungal inoculation had a better control of *S. sclerotiorum* than the application of 24 h after fungal inoculation (Chen et al., 2014; Liu et al., 2010), so this method for inoculation was

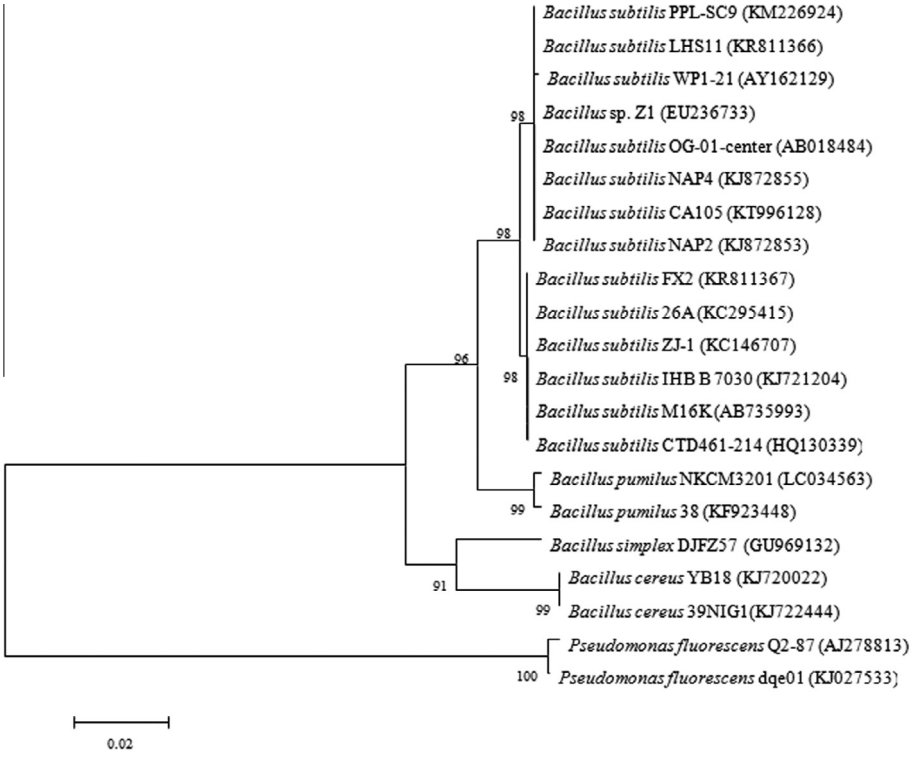

Fig. 1. Phylogenetic tree of strains LHS11 and FX2 base on maximum-likehood analysis of 16S rDNA sequences data. Bootstrap values >51% are presented above branches, and bootstrap <51% are not shown. *Pseudomonas fluorescens* Q2-87 (AJ278813) and P fluorescens dqe01 (KJ027533) represent the out-group.

Table 6
Effects of microbial inoculant on height and biomass of rapeseed plants.

Treatments	Plant height (mm)	Aboveground fresh weight (g)	Aboveground dry weight (g)	Underground fresh weight (g)	Underground dry weight (g)
A	189.26 ± 14.30^{ab}	1.5531 ± 0.20^{ab}	0.1258 ± 0.02^{ns}	0.0265 ± 0.01^{ab}	0.0051 ± 0.00^{ab}
B	204.68 ± 7.06^{a}	1.6979 ± 0.25^{a}	0.1363 ± 0.02^{ns}	0.0315 ± 0.01^{ab}	0.0060 ± 0.00^{ab}
C	217.76 ± 12.11^{a}	1.7794 ± 0.14^{a}	0.1343 ± 0.01^{ns}	0.0495 ± 0.00^{a}	0.0086 ± 0.00^{a}
D	164.60 ± 8.13^{b}	1.0234 ± 0.10^{b}	0.0926 ± 0.01^{ns}	0.0117 ± 0.00^{b}	0.0030 ± 0.00^{b}

A Single inoculant LHS11.
B Single inoculant FX2.
C Compound inoculant (LHS11 + FX2).
D Control.
Values in the table are mean ± *SE*. Different superscript letters within the same column indicate significant difference at $P < 0.05$ level by Duncan's test.
^{ns} There are no statistical differences among treatments.

Table 7
Effects of microbial inoculant on root traits of rapeseed plants.

Treatments	Total root length (cm)	Root surface area (cm^2)	Root diameter (mm)	Root volume (cm^3)
A	52.76 ± 5.67^{c}	9.11 ± 0.58^{b}	0.333 ± 0.02^{a}	0.075 ± 0.01^{ns}
B	71.01 ± 8.61^{b}	10.77 ± 0.57^{a}	0.354 ± 0.04^{a}	0.087 ± 0.02^{ns}
C	88.33 ± 2.86^{a}	10.98 ± 0.46^{a}	0.377 ± 0.02^{a}	0.079 ± 0.02^{ns}
D	56.34 ± 5.44^{bc}	8.53 ± 0.25^{b}	0.255 ± 0.01^{b}	0.056 ± 0.01^{ns}

A Single inoculant LHS11.
B Single inoculant FX2.
C Compound inoculant (LHS11 + FX2).
D Control.
Values in the table are mean ± *SE*. Different superscript letters within the same column indicate significant difference at $P < 0.05$ level by Duncan's test.
^{ns} There are no statistical differences among treatments.

chosen. In this study, the biocontrol effect of compound inoculant (LHS11 + FX2) is more obvious than single inoculant on rapeseed leaves both *in vitro* and *in vivo*. It reveals that compound inoculant (LHS11 + FX2) is very effective in controlling SSR. Strains LHS11 and FX2 can coexist, as confirmed by antagonistic reaction test, therefore the compound inoculant (LHS11 + FX2) may improve the biocontrol efficacy by interaction of two strains. Cheng et al. (2014) showed that the lesions become smaller after pretreating with YJ1 fermentation liquid, and the inhibition rate reached 80.26%. Kamensky et al. (2003) also found that *Serratia plymuthica* IC14 protected cucumber against *S. sclerotiorum* under greenhouse conditions, reducing disease incidence by 84%. Compared with the

Table 8
Effects of microbial inoculant on root length under each selected diameter class.

Treatments	Root diameter (mm)			
	0–0.5	0.5–1.0	1.0–1.5	>1.5
A	80.38 ± 10.04[a]	2.35 ± 0.40[b]	0.58 ± 0.24[ns]	0.05 ± 0.03[ns]
B	77.57 ± 10.41[a]	2.69 ± 0.31[ab]	0.66 ± 0.37[ns]	0.18 ± 0.16[ns]
C	98.50 ± 4.41[a]	3.45 ± 0.25[a]	0.32 ± 0.14[ns]	0.06 ± 0.06[ns]
D	45.61 ± 9.54[b]	2.06 ± 0.40[b]	0.26 ± 0.13[ns]	0.15 ± 0.15[ns]

A Single inoculant LHS11.
B Single inoculant FX2.
C Compound inoculant (LHS11 + FX2).
D Control.
Values in the table are mean ± *SE*. Different superscript letters within the same column indicate significant difference at $P < 0.05$ level by Duncan's test.
[ns] There are no statistical differences among treatments

biocontrol of SSR *in vivo*, biocontrol efficacy on *in vitro* leaves was more profound and it may be due to the complexity of the micro environment in the non-sterile soil. The suppression of SSR on rapeseed plants by cell suspensions of strain LHS11 indicates that the strain might produce antifungal substances which are present in the media applied to the plants. Therefore, future work needs to determine the antifungal substances that strain LHS11 produces, and to shed light on revealing the colonisation and transport of strains LHS11 and FX2 which could help us further understand the biocontrol mechanism of PGPR.

Based on the morphological, biochemical and molecular characterization, strains LHS11 and FX2 were identified as *B. subtilis*. Some strains of *B. subtilis* have made significant contribution for controlling SSR on rapeseed plants in previous studies (Gao et al., 2014; Chen et al., 2014; Kamal et al., 2016). Meanwhile, some strains of *B. subtilis* also have showed potential effectiveness for control of SSR on other crops. For example, Cazorla et al. (2007) revealed that diverse antagonistic *B. subtilis* strains isolated from healthy avocado rhizoplanes have shown promising biocontrol activity.

In this study, tested strains possess multiple plant growth promoting traits, such as P-solubilization (3.79–204.74 mg/L), IAA production (4.34–54.36 mg/L), nitrogenase activity (7.14–246.46 nmol/mL·h). Kloepper et al. (1988) reported solubilization of minerals such as phosphorus as one of the most readily available for plant growth. IAA production by microbes promoted the root growth by directly stimulating plant cell elongation or cell division (Khalid et al., 2004; Glick, 1995). PGPR that stimulate plant growth via both direct and indirect mechanisms are widely considered as alternatives to common biofertilizers or biocontrol agents (Jha and Saraf, 2015). This is in accordance with earlier studies demonstrating the plant growth promoting activities of such PGPR which has been recently reviewed by Mehta et al. (2010) and Jha and Saraf (2015). Strains LHS11 and FX2 were chosen to evaluate their plant growth promoting effects under greenhouse conditions.

In this study, an increase in the plant growth by seed bacterization has been demonstrated. The bacteria isolated from the rhizosphere hold potential in improving plant growth when applied as seed treatments (Kishore et al., 2005). Solid-based carriers provided the microenvironment and protected the transported microorganism from hostile conditions and maintain strains over an acceptable time period (Smith, 1992). The plant height, aboveground fresh weight, underground fresh weight and dry weight of plants treated with compound inoculant (LHS11 + FX2) were significantly higher than the corresponding control, and the result is similar with Ambrosini et al. (2012). Roots interact extensively with soil microorganisms which further impact on plant nutrition either by influencing nutrient availability or through plant root growth-promoting (Richardson et al., 2009). Compound inoculant (LHS11 + FX2) significantly increased total root length, root surface area and average root diameter, so the increased nutrient uptake parameters could be attributed to the enhancement of the root growth and development. According to Zak et al. (2003), plant species differ in their biochemical composition, both the quantity and quality of root exudates vary according to plant species. Thus, the composition and function of soil microbial communities must be controlled in the further research.

Further, PGPR strains LHS11 and FX2 should be safer to environment, plants and humans than fungicides. If commercialisation of strains LHS11 and FX2 are economically feasible, application of strains LHS11 and FX2 could reduce the use of fungicides and pollution of the environment. Therefore, *B. subtilis* LHS11 and FX2 are promising biological control agents and should be further investigated for control of SSR of rapeseed plant under field conditions.

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