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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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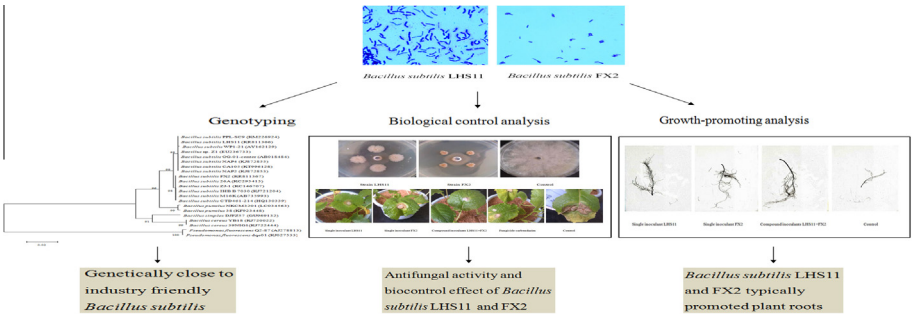
HIGHLIGHTS

- 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.

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.