Screening and Application of *Bacillus* Strains Isolated from Nonrhizospheric Rice Soil for the Biocontrol of Rice Blast

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Rice blast, caused by *Magnaporthe oryzae*, is one of the most destructive rice diseases worldwide. The aim of this study was to screen bacterial isolates to efficiently prevent the occurrence of rice blast. A total of 232 bacterial isolates were extracted from nonrhizospheric rice soil and were screened for antifungal activity against *M. oryzae* using a leaf segment assay. Strains S170 and S9 showed significant antagonistic activity against *M. oryzae* *in vitro* and in leaf disk assays, and controlled *M. oryzae* infection under greenhouse conditions. The results showed that strains S170 and S9 could effectively control rice leaf blast and panicle neck blast after five spray treatments in field. This suggested that the bacterial strains S170 and S9 were valuable and promising for the biocontrol of rice disease caused by *M. oryzae*. Based on 16S rDNA, and *gyrA* and *gyrB* gene sequence analyses, S170 and S9 were identified as *Bacillus amyloliquefaciens* and *B. pumilus*, respectively. The research also demonstrated that *B. amyloliquefaciens* S170 and *B. pumilus* S9 could colonize rice plants to prevent pathogenic infection and evidently suppressed plant disease caused by 11 other plant pathogenic fungi. This is the first study to demonstrate that *B. amyloliquefaciens* and *B. pumilus* isolated from nonrhizospheric rice soil are capable of recolonizing internal rice stem tissues.

Keywords : *Bacillus*, biocontrol, nonrhizospheric rice soil, rice blast, screening

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Rice is the most important grain crop and provides the main food energy for more than 50% of the world population every day (Food and Agriculture Organization of the United Nations, 2012). Rice blast, a widespread and destructive disease caused by *Magnaporthe oryzae* B. Couch (*Pyricularia grisea* [Cooke] Sacc.), has occurred in more than 85 countries worldwide and causes 30% of annual rice yield losses, which could feed 60 million people (Dagdas et al., 2012; Muthayya et al., 2014). *M. oryzae* can infect every tissue of rice plants, causing blast leaf and panicle neck blast, which significantly damage grain yield and quality. At present, breeding disease-resistant varieties and applying chemical pesticides are the main strategies used to control rice blast disease. Breeding new varieties takes a long time, during which the physiological race of *M. oryzae* can easily change. Moreover, chemical fungicides often cause fungicide resistance, residual toxicity and threaten to food safety. Therefore, a safe and environmentally friendly strategy for the biological control of rice blast is urgently required.

It is well known that soil bacteria can reduce the severity of disease caused by fungal pathogens and promote plant growth (He et al., 2019). Various soil bacteria such as *Bacillus* spp., *Pseudomonas* spp., and *Actinomyces* spp. have been assessed for their capacity to biocontrol rice blast in the field (Chaiham et al., 2009; Jambhulkar et al., 2018; Rais et al., 2016; Wu et al., 2018), inhibit the growth of *M. oryzae* mycelia and suppress diseases *in vitro* (Amruta et al., 2016; Kumar et al., 2017). *Bacillus* spp. belong to the phylum Firmicutes and produce spores. They are character-

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ized by an ability to biocontrol many diseases and pests, promote plant growth, yield and stress resistance, and improve the soil nutrient condition. In addition, *Bacillus* spp. have many ecological functions including acting as microbial fertilizers, restoring contaminated soil, and degrading cellulose, and are applied extensively for sustainable agricultural development.

Bacillus spp. colonize rice plants and compete with plant pathogens for colonization sites and nutrients. These species also produce antimicrobial metabolites that inhibit the growth of pathogen hyphae and conidia. Furthermore, *Bacillus* spp. dissolve pathogenic cell walls using different enzymes to reduce disease severity. *Bacillus* spp. also stimulate induced systemic resistance to promote plant resistance to disease and abiotic stress. Therefore, an increasing amount of research has focused on screening *Bacillus* spp. to obtain biological control agents. The fermentation broth or culture filtrate of *B. subtilis* strain BJ-1 was found to completely suppress the growth of *M. oryzae* on detached leaves and when applied as seed treatment against rice blast (He et al., 2019). Several *Bacillus* spp. isolated from rice leaves by Wiraswati et al. (2019) were found to inhibit the growth of *M. oryzae* using polyketide synthase and nonribosomal peptide synthetase genes. *B. subtilis* BS155 can suppress the growth of *M. oryzae* via the production of cyclic lipopeptides named fengycin BS155 (Zhang and Sun, 2018).

Bacillus strains reported in previous studies have mainly been isolated from the rhizosphere zone (Amruta et al., 2016), with few strains coming from the nonrhizospheric rice soil (soil cores without rice plants). Moreover, many reports have focused on the antifungal activity and metabolites of *Bacillus*. Rais et al. (2016) screened five *Bacillus* spp. isolated from the rice rhizosphere zone that showed strong antagonism towards *M. oryzae*. In the present study, emphasis was placed on the field activity of *B. amyloliquefaciens* and *B. pumilus* as biocontrol agents of rice blast applied through seed treatment and spraying. The main objectives were to (1) screen promising *Bacillus* species for suppression of *M. oryzae* P131 *in vitro* and on detached leaves; (2) detect the biocontrol activity of *Bacillus* agents against rice blast under greenhouse conditions and in the field; and (3) clarify the colonization abilities of S170 and S9, determine whether the growth of the hyphae and conidia of *M. oryzae* P131 is inhibited by strains S170 and S9, and detect biocontrol determinants of strains S170 and S9; and (4) provide new biocontrol agents for comprehensive treatment of rice blast.

Materials and Methods

Soil sampling of rice fields. At the sites chosen for collecting soil samples, rice had been planted for several decades and there were severe occurrences of rice blast every year. Soil samples were collected from five locations in Ningxia (38.26-38.91°N, 105.53-106.52°E), China. Ten soil samples were collected per location from nonrhizospheric rice soil after the harvesting of the rice. Soil samples from the same field were subsequently combined to make a composite sample. Samples were transported in an icebox to the laboratory to be further analyzed.

Isolation of bacteria. Each soil sample was serially diluted up to 10^{-8} with distilled water. Subsequently, 100 μ l of the most diluted soil re-suspension was spread on nutrient agar (NA) medium plates (1 l NA medium contained 10 g peptone, 3 g beef powder, 5 g NaCl and 15 g agar; the medium was made up to 1 l with distilled water, pH 7.3 ± 0.1). The plates were incubated at 30°C for 48 h. A quadrant streaking method was performed to isolate pure colonies of *Bacillus*. The pure isolates were preserved in 40% glycerol at -80°C for further processing.

Preparation of *Bacillus*, the rice blast pathogen and other phytopathogens. A virulent strain of *M. oryzae* P131 was provided by the Academy of Plant Protection, China Agricultural University. The fungus was grown on potato dextrose agar (PDA) medium (1 l contains 200 g potato, 20 g glucose, 15-20 g agar, and sterile water was added to make 1 l; pH neutral) at 28°C for 9 days and stored on PDA slants at 4°C. There were 11 strains of other phytopathogens used to detect the antimicrobial spectrum of bacteria isolated from the soil samples. The phytopathogens that were isolated in our laboratory were *Fusarium oxysporum* N16-2-1, *F. solani* N18-1-2, and *F. moniliforme* N19-2-2. The other phytopathogens, *Rhizoctonia solani* RS8, *F. oxysporum* f. sp. *niveum* M8, *Botrytis cinerea* ZDP4, *Colletotrichum gloeosporioides* ZDP21, *Alternaria alternata* BJ-A5, *A. alternata* BJ-H9, *Phytophthora parasitica* var. *nicotianae* T15, and *A. alternata* BJ-ST24, were procured from Dr. Dianpeng Zhang (Beijing Academy of Agriculture and Forestry, China). These fungi were incubated on PDA medium at 25°C for 7 days and stored on PDA slants at 4°C.

Bacillus spp. were incubated at 30°C for 24 h on NA medium plates and were cultured in Luria-Bertani (LB) medium (1 l medium contained 10 g tryptone, 5 g beef

extract, 10 g NaCl, and distilled water was added to make 1 l; pH 7.4-7.6) for 72 h at 30°C with shaking at 200 rpm. Next, the fermentation was centrifuged for 20 min at 4°C and 5,000 ×g. The centrifugation product was subsequently filtered using 0.22 µm biofilters to obtain sterile supernatant.

Detecting antagonistic activity of *Bacillus* strains using leaf segment assays. The experimental rice leaf segments, which were healthy leaves from the susceptible cultivar, Ningjing no. 43, were cut to 5 cm in length. In total, 90 rice leaf segments (30 for each replicate) were soaked in fermentation broths (10^8 colony forming units [cfu]/ml) of strain S170 or S9 for 60 min. Another 90 leaf segments were soaked in sterile water as the control group and in Lv-dikang (several *Bacillus* strains were mixed) as a positive control at a concentration of 100 times the diluting agent (Zhong Nong Lv Kang [Beijing] Biological Technology Co., Ltd., Beijing, China). After 24 h of soaking, *M. oryzae* P131 inocula with 1×10^6 sporangia/ml were sprayed onto the leaves using a spray bottle. Inoculated leaf segments were kept in a growth cabinet at 28°C. They were then exposed to 85% relative humidity (RH) and a 12 h photoperiod for 48 h before the disease index (DI) was calculated.

Disease severity was defined according to the following scale (0-9): 0 = healthy leaf, 1 = brown spot, similar in size to a needle tip; 2 = large brown spot; 3 = small, round, brown necrosis scab, 1-2 mm in diameter; 4 = classical rice blast scab, 1-2 mm in diameter, the disease covers less than 2% of the leaf area; 5 = classical rice blast scab, the disease covers less than 10%; 6 = classical rice blast scab, the disease covers from 10 to 25%; 7 = classical rice blast scab, the disease covers from 26 to 50%; 8 = classical rice blast scab, the disease covers from 51 to 75%; 9 = all leaves have died.

The DI and control efficacy were calculated according to the following formulae. $DI = \sum(\text{The respective disease scale} \times \text{The number of leaf segments for that specific scale}) / (\text{The total number of leaves} \times 9) \times 100$ (La Torre et al., 2014). The control efficacy % = $(DI \text{ of the control group} - \text{That of the treated group}) \times 100$.

Identification of strains S170 and S9. Strains S170 and S9 were identified by analyzing 16S rDNA and gyrA or gyrB gene sequences. The chloroform extraction procedure was used for DNA extraction. The bacterial universal primer pair, 27 F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 R (5'-GGTTACCTTGTTACGACTT-3'), was used to amplify the 16S rDNA gene (Kim et al., 2010). The universal primer pair was not sufficient for identifying

the two strains. Therefore, strain S170 was identified using the gyrA F (5'-CAGTCAGGAAATGCGTACGTCCTT-3') and gyrA R (5'-CAAGGTAATGCTC-CAGGCATTGCT-3') primers (Chun and Bae, 2000), and strain S9 was identified using the gyrB F (5'-TTATC-TACGACCTTAGACG-3') and gyrB R (5'-TAAATTGAAGTCTTCTCCG-3') primers (Liu et al., 2013). PCR was performed using a Taq DNA polymerase kit (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China) in 50 µl reactions. The PCR amplification contained several steps, including an initial denaturation step at 95°C for 5 min, then 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s, followed by a final extension step at 72°C for 10 min. The DNA sequences of the PCR products were blasted in the GenBank (Bethesda, MD, USA; <http://www.ncbi.nlm.nih.gov/>). The gene sequences were then analyzed with CLUSTAL 2.0 (Thompson et al., 1997) and Mega 5.0 using the neighbor-joining method (Kim et al., 1993).

The antagonistic activity of strains S170 and S9 against the 12 phytopathogens. A dual culture method was used to assess the spectrum of antagonistic activity of strains S170 and S9 against the 12 phytopathogens described in section 2.3, on PDA plates (Sha et al., 2016a). A pathogen disk (1 cm in diameter) was placed in the center of each plate (9 cm in diameter), and the antagonistic disk (0.5 cm in diameter) of the *Bacillus* strain was then put 2.0 cm away from the pathogen disk. No antagonistic disk was placed in the plate used as the control. Each treatment was replicated on 10 plates and repeated three times. All plates were incubated at 25°C under dark conditions. The antagonistic activity was assessed as the inhibition rate against mycelial radial growth. The antagonistic activity was then calculated using the following formula: $[(\text{The mycelial radial growth of the pathogen in the control} - \text{The mycelial radial growth of the pathogen inhibited by } Bacillus \text{ strains}) / \text{The mycelial radial growth of the pathogen in the control}] \times 100$.

The biocontrol ability of strains S170 and S9 in the greenhouse and field. Rice seeds of the Ningjing no. 43 variety were soaked in 1% sodium hypochlorite solution for 20 min and then washed three times with 70% ethanol and five times with sterile water. After keeping seeds in a petri dish in a growth chamber at 28°C for 2 days, the germinated seeds were transplanted into a pot in the greenhouse. When the rice plants had grown for 30 days in the greenhouse (28°C and 65% RH), each treatment (encompassing 30 rice plants each) was sprayed with 50 ml of fermentation broth generated from S170 and S9 (at 10^8 cfu/

ml). For the negative control, 30 rice plants were sprayed with sterile water. After 24 h, all test plants were sprayed with a suspension of *M. oryzae* P131 (at 10^6 cfu/ml). The disease severity of the rice leaf blast was investigated 7 days post inoculation. The experiments were repeated three times.

According to the field trial standard GB/T17980.19-2000 formulated by the Chinese Ministry of Agriculture based on the European Plant Protection Organization (EPPO) and Food and Agriculture Organization (FAO) guidelines, the control efficacies of strains S170 and S9 were detected over three successive years (2017-19) in Yaofu town, Pingluo county, China (38.91°N, 106.52°E) (Kumar et al., 2017; Sha et al., 2016a). The field trials were designed in a completely randomized block and each treatment involved four replicates. The average test area for each replicate was approximately 50 m². Rice seeds were soaked in a suspension of S170 or S9 (at 10^8 cfu/ml) for 1 h before planting, and chemical fungicides were not applied during the growing seasons.

Using a backpack sprayer, 2.5 l of S170 suspension or S9 suspension (at 10^8 cfu/ml) was sprayed on each replicate. Each replicate was sprayed five times during the growing season. The sprays were performed at the seedling stage, the tillering stage, the booting stage, the rupturing stage and the full heading stage; rice plants sprayed with water were used as the control group. Tricyclazole wettable powder (75%) was sprayed at a concentration of 1.05-1.20 kg/hm²; water 600-750 kg (Dow Agro Sciences Co., Ltd., Beijing, China) was used as a chemical control and Lvdikang was used as a biological control and applied at a concentration of 100 times the diluting agent. Disease assessment was performed, as described above, for the leaf blast and panicle neck blast, which were investigated at the tillering stage and harvest stage, respectively. For the investigation of leaf blast, individual leaves from four test areas were randomly selected. For the panicle neck blast investigation, 100 rice panicles from 20 plots in four test areas were randomly selected.

The grading standard for panicle neck blast was as follows: 0 = no panicle neck blast, grain blast or branch blast; 1 = only grain blast and branch blast, less than 5% of grains empty; 2 = only grain blast and branch blast, 6 to 10% of grains empty; 3 = only grain blast and branch blast, 11 to 20% of grains empty; 4 = only grain blast and branch blast, 21 to 30% of grains empty; 5 = panicle neck blast, grain blast and branch blast, 31 to 80% of grains empty; and 6 = panicle neck blast, more than 80% of grains empty.

The DI and control efficacy were calculated according to the following formulae. $DI = [\sum(\text{Rice blast rating} \times \text{Num-}$

$\text{ber of panicles at that rating})/(\text{Total number of rice panicles investigated} \times 9)] \times 100$. Control efficacy % = $[(DI \text{ of the control} - DI \text{ of the treatment})/DI \text{ of the control}] \times 100$.

The observed antagonistic effects of strains S170 and S9 using scanning electron microscopy (SEM).

For the SEM examination, a disk (1 cm in diameter) of *M. oryzae* P131 was placed in the center of each plate (9 cm in diameter) containing 15-20 ml PDA medium. The antagonistic disk (0.5 cm in diameter) of S170 or S9 was then placed 2.0 cm away from the pathogen disk. No antagonistic disk was placed in the plate used as the control. Each treatment was replicated on 10 plates and repeated three times. All plates were incubated at 25°C under dark conditions. After 5 days, the antagonistic zones inhibited by *B. amyloliquefaciens* S170 and *B. pumilus* S9 were cut into 2 mm strips to observe the hyphal structure of *M. oryzae* P131. The outer edges of *M. oryzae* P131 in the control plates were cut into 2 mm strips. To facilitate this analysis, all strips were fixed in 4% glutaraldehyde, dehydrated, and coated with gold (Kong et al., 2012). The hyphal structure of *M. oryzae* P131 was observed using an S-3400N (Hitachi, Tokyo, Japan) scanning electron microscope (Kong et al., 2012).

Bioactive substances produced by strains S170 and S9.

The bioactive substances produced by *B. amyloliquefaciens* S170 and *B. pumilus* S9, such as proteases, amylases, siderophores, cellulases, β -1,3-glucanases and volatile substances, were quantified. Protease and cellulose activities were determined according to protocols published by Abraham et al. (2013), Kumar et al. (2017), and Shakeel et al. (2015); β -1,3-glucanase activity was assayed using a protocol published by Nagpure et al. (2014). Siderophore production was tested for using a chrome azurol S assay published by Schwyn and Neilands (1987). Amylase activity was determined for each step using a method published by Shaldon (1956). The antifungal activities of the volatile substances were determined using a procedure published by Arrebola et al. (2010).

Relative inhibition rate % = $[(\text{The mycelium diameter for the empty control} - \text{The mycelium diameter for the test bacteria})/\text{The mycelium diameter of the empty control}] \times 100$.

Stem colonization ability of GFP-tagged S170 and S9.

Electrocompetent *Bacillus* cells were prepared as previously described by Sha et al. (2016b). The cells were grown in LB medium at 30°C. The pellets were harvested during the exponential growth phase by centrifugation at 8,000 $\times g$ for 10 min at 4°C. The pellets were subsequently washed five

times with an equal volume of cold electroporation buffer containing 0.5 M sorbitol, 0.5 M mannitol and 10% glycerol (pH 7). The green fluorescent protein (GFP) plasmid pGFP78 (100 mg) was added to the suspension of *Bacillus* cells (100 ml) and immediately exposed to a 1.8 kV electric shock. The entire electroporation process was carried out on ice. After electroporation, the cell suspension was diluted with 1 ml of LB medium containing 0.5 M sorbitol and 0.38 M mannitol and was subsequently incubated at 37°C while shaking at 160 rpm for 3 h to facilitate the expression of antibiotic resistance markers. Aliquots were spread onto LB plates containing tetracycline (10 g/ml). Cells with green fluorescence were used for the following experiments.

The GFP-tagged S170 and S9 strains were prepared in LB liquid medium in a shaker at 200 rpm at 30°C for 2 days. Cells were re-suspended in sterile water to adjust the density to 10^8 cfu/ml. The suspensions were sprayed onto the stem and leaf surface of 20-day-old G19 cultivars growing in a greenhouse. Other plants sprayed with sterile

Table 1. Biocontrol effect of isolates S170 and S9 on rice blast in leaf disk assays

Treatment ^a	Disease index ^b	Antifungal efficacy (%)
S170	7.5 ± 0.3 b	75.5 ± 0.7
S9	7.6 ± 0.2 b	74.6 ± 1.1
Lvdikang	7.1 ± 0.4 b	76.2 ± 1.1
Water control	29.6 ± 0.6 a	-

Values are presented as mean ± standard error.

^aThe concentration of the S170 and S9 inoculums was 1×10^8 colony forming units/ml.

^bThe disease index was determined 72 h after *Magnaporthe oryzae* P131 inoculation. Values are representative of three experiments, and four replicates were used for each experiment. Different letters indicate significant differences according to a Student's *t*-test ($P < 0.05$).

water were used as controls. Each treatment involved six replicates (feeding block), and the experiment was repeated twice. The stems (3 cm from the base) were collected 5 days after inoculation for all treatments. Stem tissue was sliced by cryosectioning to generate sections with a

Fig. 1. Phylogenetic tree generated based on the *gyrA* gene sequence indicating the position of strain S170 (using the neighbor-joining method) (A) and on the *gyrB* gene sequence indicating the position of strain S9 (using the neighbor-joining method) (B).

thickness of 20 mm. The slides were viewed directly using a confocal scanning laser microscope (CSLM; Leica Fluoview TCSSP8, Leica Microsystems Ltd., Wetzlar, Germany) with an excitation wavelength of 488 nm.

Statistical analysis. Each analysis was carried out using the least significant difference test ($P < 0.05$) following one-way analysis of variance (ANOVA) using SPSS ver. 19.0 software for Windows (SPSS Inc., Chicago, IL, USA).

Results

Screening *Bacillus* isolates using leaf disk assays. A total of 232 bacterial strains were isolated from nonrhizospheric rice soil. All of the isolates were screened for the ability to control rice blast using the leaf disk assay. There were 15

bacterial isolates showing fungal activity against *M. oryzae* P131 on detached leaves. In particular, isolates S170 and S9 evidently inhibited rice blast from 1 to 3 days after inoculation, especially on day 2. The DI values of leaves treated by isolates S170 or S9 were significantly lower than those of the leaves of the water control group. The control efficacy of the isolates S170 and S9 was 75.5% and 74.6%, respectively (Table 1). There were no differences in the DI between experimental isolates in the control group and the Lvdikang control group.

Identification of strains S170 and S9. Based on 16S rDNA sequence alignments, S170 (KY264994 in the National Center for Biotechnology Information [NCBI]) had a high similarity with *B. amyloliquefaciens*, while S9 (KY264956 in NCBI) was highly similar to *B. pumilus*.

Fig. 2. The mycelial growth rate of *Magnaporthe oryzae* P131 is inhibited by antagonistic bacteria. The mycelia of *M. oryzae* P131 (A); and the mycelia of *M. oryzae* P131 inhibited by strains S170 (B) and S9 (C).

Table 2. Antagonist activity of isolates S170 and S9 against various fungal plant pathogens *in vitro*

Target fungal pathogens	Host	Inhibitory activity	
		S170	S9
<i>Magnaporthe oryzae</i> P131	Rice	++++	++++
<i>Fusariumoxysporum</i> N16-2-1	Potato	+++	+
<i>Fusarium solani</i> N18-1-2	Potato	+	–
<i>Fusarium moniliforme</i> N1-2-2	Potato	+	–
<i>Rhizoctonia solani</i> RS8	Wheat	++	++
<i>Botrytis cinerea</i> ZDP4	Tomato	+	+
<i>Colletotrichum gloeosporioides</i> ZDP21	Strawberry	++++	+
<i>Alternaria alternata</i> BJ-A 5	Apple	+++	++
<i>Alternaria alternata</i> BJ-ST24	Strawberry	++++	+++
<i>Alternaria alternata</i> BJ-H9	Chinese flowering crabapple	++++	+++
<i>Fusarium oxysporum</i> f. sp. <i>niveum</i> M8	Watermelon	+++	+
<i>Phytophthora parasitica</i> var. <i>nicotianae</i> T15	Tobacco	+++	+

+, ++, +++, and ++++ represent relative mycelial growth inhibition rates for each fungal colony on potato dextrose agar medium. Inhibition rates were recorded as follows: +, 50-60%; ++, 61-70%; +++, 71-80%; and ++++, >80%. – represents no inhibition rates.

Sequencing the *gyrA* and *gyrB* genes revealed that S170 had 100% correspondence with *B. amyloliquefaciens* NAU-B3 and S9 had 100% correspondence with *B. pumilus* ATCC7061. Phylogenetic trees were constructed using the *gyrA* gene sequence for S170 (Fig. 1A), and the *gyrB* gene sequence for S9 (Fig. 1B). The results clearly showed that strain S170 clustered with *B. amyloliquefaciens* strains while strain S9 clustered with *B. pumilus* strains. Taken together, these results suggest that S170 is a *B. amyloliquefaciens* strain, while S9 is a *B. pumilus* strain.

The antagonistic activity of strains S170 and S9 against 12 phytopathogens. Strains S170 and S9 significantly inhibited the growth of the mycelia of *M. oryzae* P131 on PDA medium plates (Fig. 2). Moreover, both strains exhibited broad-spectrum antagonistic activities against 11 other pathogens in vitro (Table 2). Strains S170 and S9 evidently suppressed the growth of *Fusarium oxysporum*, *F. solani*, *F. moniliforme*, *Rhizoctonia solani*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Alternaria alternata*, *F. oxysporum*

Table 3. Control efficacy of S170 and S9 against rice blast under greenhouse conditions

Treatment ^a	Disease index ^b	Control efficacy (%)
S170	5.1 ± 1.2 b	76.9 ± 0.5
S9	5.5 ± 0.8 b	75.1 ± 0.9
Lvdikang	5.0 ± 1.1 b	77.4 ± 1.1
Water control	22.1 ± 2.4 a	-

Values are presented as mean ± standard error.

^aThe concentration of the S170 and S9 inoculums was 1×10^8 colony forming units/ml.

^bThe disease index was determined 7 days after *Magnaporthe oryzae* P131 inoculation. Values are representative of three experiments, and four replicates were used for each experiment. Different letters indicate significant differences according to the Student's *t*-test ($P < 0.05$).

f. sp. niveum, and *Phytophthora parasitica* var. *nicotianae*, which cause rice sheath blight, tomato gray mold, strawberry anthracnose, apple leaf blight, begonia leaf blight, watermelon wilt disease and tobacco black shank. Isolate S170 showed a broader antagonistic spectrum than isolate S9.

Table 4. Control efficacy of strains S170 and S9 against rice leaf blast in the field

Treatment ^a	2017		2018		2019	
	Disease index	Control efficacy (%) ^b	Disease index	Control efficacy (%) ^b	Disease index	Control efficacy (%) ^b
S170	8.5 ± 0.5 b	73.2 ± 1.1	2.9 ± 0.8 b	91.1 ± 1.7	7.1 ± 1.1 b	84.8 ± 1.2
S9	9.4 ± 1.2 b	70.6 ± 1.3	7.1 ± 1.4 b	79.3 ± 1.6	13.1 ± 2.7 b	71.9 ± 3.4
Biological control	7.8 ± 0.3 b	75.6 ± 0.7	4.1 ± 1.7 b	88.7 ± 2.4	16.4 ± 1.3 b	64.9 ± 1.9
Chemical control	8.4 ± 0.2 b	73.5 ± 2.1	9.7 ± 2.5 b	72.2 ± 2.1	11.8 ± 2.2 b	74.7 ± 1.9
Water control	31.8 ± 0.3 a	-	36.2 ± 2.1 a	-	46.7 ± 2.1 a	-

Values are presented as mean ± standard error.

^aThe concentration of the S170 and S9 inoculums was 1×10^8 colony forming units/ml.

^bThe disease index of leaf blast was determined at the tillering stage of rice. Values are representative of five experiments, and four replicates were used for each experiment. Different letters indicate significant differences according to the Student's *t*-test ($P < 0.05$).

Table 5. Control efficacy of strains S170 and S9 against rice panicle blast in the field

Treatment ^a	2017		2018		2019	
	Disease index	Control efficiency (%) ^b	Disease index	Control efficiency (%) ^b	Disease index	Control efficiency (%) ^b
S170	11.8 ± 0.7 b	74.8 ± 0.4	14.8 ± 0.7 b	72.0 ± 1.1	5.3 ± 0.7 b	78.4 ± 2.3
S9	13.3 ± 0.3 b	71.6 ± 0.3	15.3 ± 0.3 b	71.1 ± 0.9	6.8 ± 3.8 b	72.2 ± 0.9
Biological control	12.3 ± 0.7 b	76.3 ± 0.4	15.1 ± 1.8 b	71.5 ± 1.5	6.6 ± 1.3 b	73.1 ± 1.5
Chemical control	12.1 ± 0.5 b	74.2 ± 1.1	15.4 ± 1.2 b	70.9 ± 1.3	6.1 ± 0.9 b	75.1 ± 1.3
Water control	46.9 ± 3.1 a	-	52.9 ± 2.3 a	-	24.5 ± 1.3 a	-

Values are presented as mean ± standard error.

^aThe concentration of the S170 and S9 inoculums was 1×10^8 colony forming units/ml.

^bThe disease index of panicle neck blast was determined at the yellow ripening stage of rice. Values are representative of five experiments, and four replicates were used for each experiment. Different letters indicate significant differences according to the Student's *t*-test ($P < 0.05$).

The biocontrol activity assays of strains S170 and S9.

Under greenhouse conditions, strains S170 and S9 exhibited strong antifungal activity following the controlled *M. oryzae* infection (Table 3). There was a 5.2-fold reduction in DI in the S170-treated group and a 4.2-fold reduction in DI in the S9-treated group compared with the water control. There were no differences in DI between the control group and the Lvdikang control group.

The ability of strains S170 and S9 to suppress rice blast

were tested in the field between 2017 and 2019 (Tables 4 and 5). In 2017, a small-scale experiment was conducted and both strains evidently suppressed *M. oryzae* infection. To confirm their biocontrol ability, both a small-scale and larger scale experiments were conducted in 2018 and 2019. The results of these 3-year studies revealed 8.1- and 3.8-fold reductions in DI in the S170-treated group compared with the water control group for leaf blast and panicle neck blast, respectively. Treatment with S9 resulted in a 4.2- and

Fig. 3. Scanning electron microscope analysis of *Magnaporthe oryzae* P131. (A-C) The normal hyphae and conidia of *M. oryzae* P131. (D-F) The hyphae and conidia of *M. oryzae* P131 inhibited by *Bacillus pumilus* S9. (G-I) The hyphae and conidia of *M. oryzae* P131 inhibited by *B. amyloliquefaciens* S170. CN represents the conidia of *M. oryzae* P131, red arrows point to conidia, and the boxes surround hyphae of typical morphology.

Table 6. Bioactive substances produced by S170 and S9

Strains	Protease	Amylase	Cellulose	Siderophore	Glucanase	Volatile substances
S170	+	+	+	–	–	+
S9	+	–	+	+	–	+

+, the production of the biocontrol-related substance by the test strain; –, a lack of production of the biocontrol-related substance by the test strain.

Fig. 4. The inhibitory activity of volatile substances produced by test strains. The normal mycelia of *Magnaporthe oryzae* P131 (A); and the mycelia of *M. oryzae* P131 inhibited by volatile substances produced by strains S9 (B) and S170 (C).

3.5-fold reduction in DI, respectively. It was observed that disease injuries were effectively controlled following application of either strain along with both chemical and biological fungicides during the growing season. These results suggest that both strains can effectively control *M. oryzae* infection in field conditions.

SEM analysis of *M. oryzae* P131 inhibition by strains S170 and S9. The hyphae of *M. oryzae* P131 that were not treated with antagonistic bacteria were completely tubular in shape and were morphologically normal when observed by SEM (Fig. 3A-C). Hyphae that were treated with *B. pumilus* strain S9 displayed swelling and partial distortion (Fig. 3D-F). The hyphae treated with *B. amyloliquefaciens* strain S170 showed shrinkage, partial distortion, roughness, hollowness and wrinkling of the surface, indicating overall morphological abnormality (Fig. 3G-I).

The conidia of *M. oryzae* P131 that were not exposed to antagonistic bacteria formed morphologically normal appressoria, as observed under SEM (Fig. 3B). After exposure to *B. pumilus* strain S9 and *B. amyloliquefaciens* strain S170, the cell walls of the conidia appeared to be severely degraded (Fig. 3D and G).

Overall, the cells of *M. oryzae* P131 treated with *B. pumilus* strain S9 and *B. amyloliquefaciens* strain S170 exhibited abnormal morphology and cellular disorganization, suggesting that S9 and S170 degrade the cell wall, destroy

the cell membrane and damage cellular organelles.

Detection of bioactive substances of strains S170 and S9. Based on the transparent zone observed after each treatment, it was possible to analyze biocontrol determinants (Table 6). Following this analysis, *B. amyloliquefaciens* S170 and *B. pumilus* S9 were observed to produce proteases, amylases, cellulases and volatile substances. Strain S9 also produced siderophores. The relative inhibition ratios of the volatile substances produced by strains S170 and S9 were 56.25% (Fig. 4B) and 61.11% (Fig. 4C), respectively.

Colonization ability of S170 and S9. To detect the colonization abilities of S170 and S9, GFP-tagged strains were sprayed onto stems of rice planted in a greenhouse. These plants were examined by CSLM 7 days later. The results revealed that the GFP-tagged cells were easily observed between the epidermis and ground tissues of the treated stems (Fig. 5C and E). The parenchymatous tissues of the stems were observed using fluorescence microscopy (Fig. 5D and F), whereas no fluorescence was observed in the control stems (Fig. 5A and B). These results suggest that both soil bacterial strains were capable of colonizing rice stems.

Discussion

At present, some strains have been applied to control

Fig. 5. Confocal microscopy images of rice stems inoculated with GFP-tagged strains. Transverse and longitudinal sections of the rice stems of the control group treated with sterile water (A, B); and of the rice stems treated with GFP-tagged S9 (C, D) and GFP-tagged S170 (E, F).

rice blast for sustainable agricultural development. These strains include *B. subtilis*, *B. cereus*, *B. amyloliquefaciens*, *B. pumilus*, *B. licheniformis*, and *Paenibacillus polymyxa*. Due to their evident ability to control many plant pathogens, promote plant growth and enhance crop yield, plant-growth-promoting rhizobacteria have become the main research focus for an increasing number of plant pathologists. Rais et al. (2016, 2018) screened *Bacillus* strains isolated from the rice rhizosphere in Pakistan, such as KEF-5, KEF-

7, KEF-12, KEF-17, and KEF-18, which showed strong antifungal activity towards to *M. oryzae* and enhanced rice yield. *B. subtilis* strain UASP17, isolated from the rice rhizosphere in India by Kumar et al. (2017), evidently reduced blast severity. There are many reports about *Bacillus* spp. isolated from the rhizosphere that act against rice blast. However, few studies have reported on the strains isolated from nonrhizospheric rice soil.

In order to enrich biocontrol sources, our team isolated

Bacillus spp. with high control activity against *M. oryzae* and other plant pathogens from nonrhizospheric soil from rice fields in Northwest China. In a previous study, 17 out of 232 soil isolates exhibited potential to stimulate rice seedling growth; the same isolates promoted grain quality at the harvest stage (Sha et al., 2018). In the current study, a leaf disk assay revealed that two isolates showed significant promise in suppressing disease severity following *M. oryzae* infection under field conditions. These isolates, S170 and S9, could therefore be potentially used for the biocontrol of *M. oryzae*. Their suppressive activity suggests a prospect for practical applications in the sustainable development of rice farming.

Based on 16S rDNA, and *gyrA*, and *gyrB* gene sequence analyses, strains S170 and S9 were identified as *B. amyloliquefaciens* and *B. pumilus*, respectively. Strain S9 was identified by amplifying the *gyrA* and *gyrB* genes, but the *gyrA* gene amplification failed. Both of these species also produce bioactive compounds (Chen et al., 2016) and promote plant growth. Until now, *B. amyloliquefaciens* and *B. pumilus* have been considered to be environmentally friendly agents with effective inhibition activity against numerous plant pathogens.

In the present study, *B. amyloliquefaciens* S170 and *B. pumilus* S9 exhibited broad-spectrum antibacterial activity. The plant pathogens studied cause disease in different plants including potato, wheat, rice, watermelon, strawberry, apple, begonia and tobacco. The application of *B. amyloliquefaciens* S170 had a greater biocontrol ability than that of *B. pumilus* S9.

Bacillus species can indirectly inhibit phytopathogens through biocontrol strategies including the repossession of pathogenic iron by siderophores, the production of hydrolytic enzymes to degrade pathogenic cell walls, and the production of antibiotics to interfere with pathogenic respiration (Ahmad et al., 2017; Li et al., 2014; Li et al., 2017; Qi et al., 2017; Zhang and Sun, 2018). Huang et al. (2020) isolated *B. cereus* HS24 from a rice farm; *B. cereus* HS24 could significantly reduce conidium germination by interfering with the *M. oryzae* Ca²⁺ signaling pathway. SEM analysis revealed that *B. amyloliquefaciens* S170 and *B. pumilus* S9 degraded the hyphae and conidial cell walls of *M. oryzae* P131. The same strains caused partial mycelial morphology inflation or crimp, and few appressoria and germinal tubes in *M. oryzae*. It is possible that hydrolytic enzymes and antibiotic production in strains S170 and S9 degraded cell walls and inhibited pathogenic growth. It is not clear what antibiotics *B. amyloliquefaciens* S170 and *B. pumilus* S9 produce, and a follow-up study will be performed to purify and study the characteristics of the me-

tabolites of the two strains.

Bacillus spp. can produce enzymes such as chitinase, β -1,3-glucanase and peroxidase, that can dissolve the cell wall of *M. oryzae*, or suppress conidium germination and appressorium formation. For example, *B. subtilis* NSRS 89-24 has been shown to produce β -1,3-glucanase, and thus, could significantly inhibit the growth of *M. oryzae* (Leelasuphakul et al., 2006). Meanwhile, *B. subtilis* KB-1122 produced tyrosine, serine and threonine kinases to cause germ tube abnormality, cell wall rupture, and mycelium collapse in *M. oryzae* (Zhang et al., 2014). In the present study, *B. amyloliquefaciens* S170 also produced proteases, amylases and cellulases. *B. pumilus* S9 produced siderophores, proteases, amylases and cellulases. However, the latter strain did not form biofilms to enhance colonization in rice plants. After following the two-sealed-base-plates method, the results indicated that volatile substances isolated from strains S170 and S9 exhibited antifungal activity towards *M. oryzae*.

Plant colonization by bacteria is deemed a key characteristic in the interaction between these bacterial species and pathogens (Huang et al., 2011). Ji et al. (2014) reported that *B. subtilis* CB-05, which is a gram-positive diazotrophic endophytic bacterium, can effectively colonize rice roots. Liu et al. (2006) reported that *B. megaterium* C4, which is a gram-negative diazotrophic rhizospheric bacterium, can also colonize rice roots. The colonization abilities of GFP-tagged S170 and S9 in the rice stem were demonstrated by confocal observation. These observations suggested that the bacteria isolated from rice soil could successfully recolonize internal tissues to protect the rice plant from infection. Because strains S170 and S9 were isolated from nonrhizospheric rice soil, it is necessary to determine their ability to colonize rice roots and the effect they have on the microbial community of nonrhizospheric rice soil. The microbial-culture method and high-throughput sequencing should now be implemented to study the culturable and uncultured microorganism diversity of nonrhizospheric rice soil treated by strains S170 and S9.

In conclusion, two *Bacillus* strains isolated from nonrhizospheric rice soil were studied to analyze their capacity to recolonize internal rice stem tissues and effectively suppress the growth of *Fusarium*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Alternaria alternata*, and *Phytophthora parasitica* among others. Both strains are biocontrol agents of *M. oryzae* in rice.

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