J Plant Dis Prot (2017) 124:131–141
DOI 10.1007/s41348-017-0080-1

ORIGINAL ARTICLE

Biocontrol activities of bacteria from cowdung against the rice sheath blight pathogen

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Received: 18 July 2016 / Accepted: 20 February 2017 / Published online: 25 February 2017

Abstract To discover bacteria with potential biocontrol activity against the rice sheath blight (RSB) pathogen, *Rhizoctonia solani,* cowdung (CD) associated bacteria were screened for antifungal activity a dual-culture method. Five potential biocontrol bacteria were identified to species-level based on their colony morphology, physiology, biochemical characteristics, utilization of carbon sources, micromorphology and 16S rRNA sequences. The ability of two selected strains to inhibit RSB was evaluated in vivo and in vitro. The median effective concentration (EC_{50}) of crude extract from *Streptomyces cochorusii* strain NF0919 (NF0919) culture filtrate was 1.3 μg ml^{-1}, lower than the EC_{50} of Jinggangmycin (a commercial antifungal agent widely used in China). At a concentration of 25.0 μg ml^{-1}, the crude extract completely inhibited mycelial growth of *R. solani.* The field biocontrol efficacy after spraying 7 days in 2013 and in 2014 was 78.4 and 98.1% with crude extract from NF0919 culture filtrate and 71.1 and 94.2% with fresh cells of *Bacillus amyloliquefaciens* strain SB177 (SB177). Results from the 2-year field experiment suggested that the crude extract from NF0919 culture filtrate or fresh cells of SB177 provided better disease control than other fungicides (Jinggangmycin and/or Kresoxim-methyl). *S. cochorusii* strain NF0919 and *B. amyloliquefaciens* strain SB177 have good potential for field application and commercial use against the RSB pathogen.

Keywords Biological control · Cowdung microflora · Identification · Rice sheath blight

Introduction

Rice sheath blight (RSB), caused by *Rhizoctonia solani* kühn, is one of the main rice diseases worldwide [28, 35, 54]. Yield losses range from approximately 2.5–50% [46]. *R. solani* survives in soils as sclerotia, so it is very difficult to control effectively with chemical fungicides [12, 13, 48]. In the long run, chemical fungicides spaying also will have adverse environmental impact. Efficient and environmentally benign agents are urgently required to control RSB.

Breeding disease-resistant rice cultivars is believed to be one of the most promising approaches to control the disease. However, no rice cultivar has been found completely resistant to the soilborne fungus so far [5, 69]. Biocontrol of RSB has been reported and well documented. Biological control of sheath blight can be achieved by using antagonistic *Pseudomonas* spp. [38–40, 47, 56], *Bacillus* spp. [9, 10, 30, 42, 59, 60, 63, 67, 68], *Trichoderma* spp. [49, 53, 57], and antifungal metabolites produced by *Streptomyces* spp. [2, 21, 31, 43, 44, 50, 62, 65].

Despite the abundant biocontrol reports, few commercial formulations of biocontrol agents have been used successfully to control the RSB. Environmental constraints (ultraviolet light, different types soil, soil or air humidity, temperature) and disadvantageous formulation characteristics (short shelf-life, uncertain content, slow activity) have limited the use of biocontrol formulations with fresh

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cells (or spores), especially for field applications. To our knowledge, the most successful antifungal antibiotics for control of RSB in China and Japan, respectively, are Jinggangmycin from *Streptomyces hygroscopicus* var. jinggangensis 5008 [50] and Validamycin from *S. hygroscopicus* var. *limoneus* [21]. Jinggangmycin has been used for 35 years in China and has become the sole antibiotic used to control sheath blight. Some reports have noted a decline in the field control efficiency of Jinggangmycin and some tolerance in isolates of *R. solani* [19, 66].

Our investigation was carried out to discover more antifungal biocontrol resources. The purpose of this study was to (a) screen and identify the potential biocontrol ability of bacteria cultivated from CD, (b) find new bacteria able to prevent RSB, and (c) evaluate the potential biocontrol agents (fresh spore suspensions of *Bacillus* spp. and crude extracts of metabolites from *Streptomyces* spp.) in vivo and in vitro conditions.

Materials and methods

Isolation and selection of antagonistic bacteria from cowdung

Fresh CD was sampled from lactating cows and brought in sterile plastic bags to the Microbiology Laboratory of Zhenjiang Institute of Agricultural Science (Zhenjiang, Jiangsu Province of east China). Ten-gram samples of CD were transferred separately to sterile Erlenmeyer flasks containing 90 ml sterile PBS solution (PBS; containing 1.2 g Na_2HPO_4, 0.18 g NaH_2PO_4, 8.5 g NaCl, made up to 1 l with distilled water, pH 7.2). The samples were shaken for 30 min at room temperature. The suspensions were filtered through doubled, sterile gauze prior to isolation of bacteria. The filtered solutions were serially diluted to 10^{-1}–10^{-5} with sterile PBS, and 0.1 ml of each dilution was spread on R2A medium (R2A; containing 0.75 g peptone, 0.50 g dextrose, 0.30 g dipotassium phosphate, 0.50 g starch, 0.50 g yeast extract, 0.30 g sodium pyruvate, 0.25 g tryptone, 0.024 g magnesium sulfate, and 15.00 g bacteriological agar, made up to 1 l with distilled water and adjusted to pH 7.2) and on Gause's synthetic media to screen bacteria and actinomycetes, respectively. Three replicates (Petri plates) were prepared for each dilution and each incubation temperature. The plates were incubated for 3–5 days at 20 °C, 25 °C, or 30 °C to assay for a wide spectrum of bacteria. The antagonism of all bacterial isolates was checked with respect to their ability to suppress the growth of *R. solani* ZJ-1 (provided by Jiangsu Academy of Agricultural Science), inoculated on Waksman agar (WA; containing 5 g proteose-peptone, 10 g glucose, 3 g meat extract, 5 g NaCl, 20 g agar, made up to 1 l with distilled water and adjusted to pH 6.8). After 5 days of incubation at 25 °C, zones of inhibition were measured as described [4]. Eleven strains of bacteria (JR08, Mg116, RM09, SB177, NF0912, NF0919, NF0931, NF0939, NF0945, NF0974, NF0998) were finally selected based on their antagonistic activity toward fungi (measurement of inhibition zones). All the antifungal isolates were purified twice and then stored at −80 °C in nutrient broth containing 15% glycerol for further use.

Elucidation of antifungal spectrum of the selected antagonists

A dual-culture plate method was used to detect the antifungal spectrum of the selected antagonists. The antifungal activity of all bacterial isolates was checked by examining their ability to suppress the growth of the following phytopathogens from the main crops in Jiangsu province: *Penicillium expansum* JR21 (causes apple blue mold), *Gloeosporium fructigenum* XX43 (causes anthracnose of grape), *Rhizopus nigricans* GS84 (causes strawberry black mold rot), *Gibberella zeae* NA49 (causes *Fusarium* head blight on wheat), *Fusarum oxysporium* SW2 (causes strawberry wilt), *Botryosppuaeria berengeriana* YN19 (causes ring rot of apple), *Fusarium oxysporum* HB16 (causes cotton fusarium wilt), *Magnaporthe grisea* 04-025-4 (causes rice blast), *Glomerella cingulata* DS11 (causes apple anthracnose), *F. oxysporum* DT04 (causes watermelon wilt), *Verticillium dahliae* XJ37 (causes cotton verticillium wilt), *Botrytis cinerea* BC08 (causes grape gray mold), *R. solani* ZJ-1 (causes RSB), and *Sclerotinia sclerotiorum* JY20 (causes white mold of canola). The phytopathogens were provided by Jiangsu Academy of Agricultural Science. All the phytopathogens were inoculated in separate plates on WA. After 5 days of incubation at 25 °C, zones of inhibition were measured as described [4].

Bacterial characterization based on production of hydrolytic enzymes and secondary metabolites

Production of cell wall degrading enzymes and antifungal secondary metabolites are common mechanisms that bacteria use to inhibit fungal growth. To better characterize the antifungal isolates, their potential production of hydrolytic enzymes and secondary metabolites was studied. Previously described methods were used to assess chitinolytic activity in minimal medium [11], siderophore production [51], amylase activity [14], cellulase activity [16], phosphorus dissolution [25], and nitrogen fixation [22]. Protease activity indicated by casein degradation was determined from distinct zones of clearing in skim milk agar (50 ml sterilized skimmed milk mixed at 55 °C with 50 ml of 1/5

Waksman medium and 2% agar) after 3 days' incubation at 30 °C.

Identification of the selected isolates

Five strains of the selected antifungal bacteria were identified taxonomically based on colony morphology, physiological and biochemical characteristics as described in *Bergey's Manual of Systematic Bacteriology* [15], utilization of carbon sources [17], micromorphology [7], and 16S rRNA gene sequences [64].

Bioassay of antifungal activity of *S. cochorusii* strain NF0919

For detection of bioactive metabolites, a loopful of cultured *S. cochorusii* strain NF0919 was inoculated in Gause's synthetic broth and incubated on a rotary shaker (200 rpm) at 30 °C as a seed culture. After 48 h of incubation, the seed culture (10% V/V) was transferred to a 30-l fermentor (Zhenjiang Dongfan GUS-30), containing 18 l optimized fermentation medium (potato starch 8.5%, cottonseed protein 2.2%, $CaCO_3$ 0.1%, K_2HPO_4 0.05%, and $MgSO_4$ 0.05%, W/V). The culture medium was agitated at 360 rpm, and the aeration flow was maintained at 3.2 air-volume/liquid-volume/minute (VVM). The fermentation was carried out for 5 days at 30 °C. The fermented culture was centrifuged at 10,000 rpm for 10 min, and the supernatant was filtered through a 0.25 μm filter. To obtain the crude extract, the culture filtrate was extracted twice with ethyl acetate and concentrated to dryness under a vacuum at 35 °C.

The crude extract (dissolved in a minimal volume of dimethyl sulfoxide) and 60% Jinggangmycin (as a positive control) were diluted to 0.78125, 1.5625, 3.125, 6.25, 12.5, 25.0, and 50.0 mg l^{-1} and mixed with molten WA medium, respectively. Ten milliliters of each treatment mixture was poured into sterile Petri plates (7 cm diameter). Agar plugs (5 mm diameter) taken from a 3-day-old WA culture of *R. solani* were placed on the WA plates as inocula. All the plates were incubated in dark at 25 °C. A culture of *R. solani* on WA without an inhibitor was used as negative control. Triplicate plates were maintained for each treatment. Mycelial growth inhibition of *R. solani* was assessed as percent mycelial growth inhibition relative to the negative control after 60 h incubation.

Field trials of RSB control

A field experiment was carried out at the Zhenjiang Institute of Agriculture Science of Jiangsu province (eastern China) to evaluate the efficacy of a fresh cell formulation of *B. amyloliquefaciens* strain SB177 and a crude extract of culture filtrate from *S. cochorusii* strain NF0919 for the suppression of RSB. Five treatments were tested: (1) 5% (W/V) Jinggangmycin (generously provided by TongLu Biochemical Company, Zhejiang, China), 375.0 g a.i. ha^{-1} (500.0 mg a.i. l^{-1}); (2) 50% Kresoxim-methyl (a chemical fungicide), 225.0 g a.i. ha^{-1} (300.0 mg a.i. l^{-1}); (3) fresh cells of SB177 (1.0–1.2 × 10^9 CFU ml^{-1}), 7.5 l ha^{-1} (1.0–1.2 × 10^7 CFU ml^{-1}); (4) crude extract of strain NF0919 culture filtrate, 125.0 g a.i. ha^{-1} (167.0 mg a.i. l^{-1}); and (5) water alone, 750.0 l ha^{-1} (untreated control). A rice field known to be naturally infested with *R. solani* (located in the Zhenjiang Institute of Agricultural Science test field) was divided into 15 plots; each plot was 20.0 m^2. The planted rice cultivar was Nangeng 44, which is highly sensitive to *R. solani*. Each treatment included three replicates arranged in a randomized complete block design. No other fungicides were applied to the experimental plots. All other treatments, such as herbicides and fertilizers, were used in accordance with standard farm practices.

An inoculum of experimental bacteria (SB177) was prepared by growth in LB medium (LB; containing 10 g tryptone, 5 g yeast extract, 10 g NaCl, made up to 1 l with distilled water and adjusted to pH 7.5) for 24 h at 30 °C, 200 rpm, and adjusted to give a final cell concentration of 1.0–1.2 × 10^9 CFU ml^{-1}. For field application, the suspension in LB was diluted with sterile-distilled water to 1.0–1.2 × 10^7 CFU ml^{-1}. A crude extract of culture filtrate from NF0919 was produced as described above. The five treatments were sprayed with 750.0 l ha^{-1} at the rice booting stage. This field experiment was performed in 2013 and repeated in 2014.

Based on the percentage of stems with disease symptoms among 200 randomly selected rice plants, disease incidence of RSB was determined after treatment 7, 14, and 21 days, respectively. Severity of RSB was rated on a 0–9 scale using the Standard Evaluation System for rice developed by the International Rice Research Institute, Manila, Philippines [20].

Disease severity and control efficacy were calculated as follows:

Disease severity = [∑(the number of diseased plants in each scale × representative value of each scale)/(total number of plants investigated × the value of the highest scale)] × 100.

Control efficacy = [(increase in disease severity in the control − increase in disease severity in the treatment)/increase in disease severity in the control] × 100.

Increase in disease severity = (treatment disease severity − basic disease severity) × 100. Basic disease severity refers to the disease severity before treatments. Data were subjected to one-way analysis of variance and compared using Duncan's multiple range test (DMRT) at $P < 0.01$.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences from five isolates were deposited in GenBank with accession numbers HM989896, HM989897, HM989898, HM989899, and HM989900.

Results

Screening of antagonistic bacteria against *R. solani*

The count of culturable bacteria in the fresh cowdung ranged from 6.6×10^5 to 1.4×10^6 CFU (cells g^{-1} fw). In total, 342 bacteria isolates were obtained and checked for antagonism against *R. solani* in an in vitro dual-culture plate assay. Forty-two (12.3%) of the isolates were antagonistic to *R. solani*. Of these antagonists, eleven isolates (NF0919, NF0912, NF0931, NF0939, NF0945, NF0974, NF0998, JR08, Mg116, RM09, and SB177) were selected for further studies based on the diameter of the inhibition zone (≥5 mm).

Elucidation of antifungal spectrum of eleven antagonists

Eleven antagonists (Table 1) were tested for inhibition of the 14 main phytopathogens of field crops and orchards in the Jiangsu province of China. Eight isolates (NF0912, NF0919, NF0939, NF0945, NF0998, Mg116, RM09, and SB177) were antagonistic to all 14 of the phytopathogens, producing distinct, wide zones (ranging from 1 to 20 mm) of inhibition of pathogen mycelium. Five of the antagonists (NF0919, JR08, Mg116, RM09, and SB177) showed the most effective inhibition of *R. solani*, with inhibition zones more than 15 mm.

Characterization of bacterial production of hydrolytic enzymes and secondary metabolites

Among the 11 selected antagonists (Table 2), no isolates expressed chitinolytic activity, inorganic phosphate dissolving activity, or organic phosphate solubilizing activity. All 11 isolates expressed proteolytic activity and amylolytic activity. Only three antagonists (SB177, RM09, and Mg116) showed siderophore activity and nitrogen fixing activity, and all seven selected actinomycetes (NF0912, NF0919, NF0931, NF0939, NF0945, NF0974, and NF0998) expressed cellulolytic activity.

Identification of five selected strains

Based on the antifungal activity and enzyme activity, five bacteria (JR08, Mg116, RM09, SB177, and NF0919) were chosen for taxonomic identification, and for the subsequent in vitro and field assays. Three strains (Mg116, RM09, and SB177) were identified as strains of *Bacillus amyloliquefaciens* according to the method described in *Bergey's Manual of Systematic Bacteriology* [15] on the basis of

Table 1 Inhibition activity produced by selected antagonists against phytopathogens

Strain	Antagonistic activity toward[a]													
	P.e.	G.f.	R.n.	G. z.	F.o.f.	B.b.	F.o.v.	M.g.	G.c.	F.o.n.	V.d.	B.C.	R.s.	S.s.
NF0912	++	++	++	++	+	+++	+	+	++	+	++	++	+++	++
NF0919	+++	++++	++++	++	++	++++	++	+++	++	++	+++	+++	++++	++++
NF0931	+	−	++	−	+	−	−	−	+	−	−	+	++	−
NF0939	+	++	++	+	+	++	+	+	+	+	++	++	+++	+
NF0945	++	++	++	++	+	++	+	+	++	++	++	++	++	++
NF0974	−	−	+	−	−	−	−	−	+	−	+	+	++	++
NF0998	++	++	++	++	+	+++	+	+	++	+	++	+++	+++	++
JR08	+	+	+	+	−	−	−	−	−	−	−	−	++++	−
Mg116	++	+++	++	++	+	++++	++	++	+++	++	++	+++	++++	+
RM09	++	+++	+++	++	+	++++	++	++	++	++	++	+++	++++	+
SB177	+	++	++	+	+	++++	++	++	++	++	++	++	++++	+++

[a] Antagonism toward these 14 species (*P.e.* = *Penicillium expansum* JR21, *G.f.* = *Gloeosporium fructigenum* XX43, *R.n.* = *Rhizopus nigricans* GS84, *G.z.* = *Gibberella zeae* NA49, *F.o.f.* = *Fusarium oxysporum* SW2, *B.b.* = *Botryospuaeria berengeriana* YN19, *F.o.v.* = *Fusarium oxysporum* HB16, *M.g.* = *Magnaporthe grisea* 04-025-4, *G.c.* = *Glomerella cingulata* DS11, *F.o.n.* = *Fusarium oxysporum* DT04, *V.d.* = *Verticillium dahliae* XJ37, *B.c.* = *Botrytis cinerea* BC08, *R.s.* = *Rhizoctonia solani* ZJ-1, *S.s.* = *Sclerotinia sclerotiorum* JY20) was determined by dual-culture assay. Assessment results indicate the width of the zone of inhibition as follows: +, represents 0–5 mm; ++, represents 5–10 mm; +++, represents 10–15 mm; ++++ , represents >15 mm

Table 2 Antifungal mechanisms of selected antagonists in vitro

Isolates	Hydrolytic enzymes[a]							Metabolite
	Chitinases	Proteases	Cellulases	Amylase	OPA[b]	NPA[b]	NFb[b]	Siderophores
NF0912	−	+	+	+	−	−	−	−
NF0919	−	+	+	+	−	−	−	−
NF0931	−	+	+	+	−	−	−	−
NF0939	−	+	+	+	−	−	−	−
NF0945	−	+	+	+	−	−	−	−
NF0974	−	+	+	+	−	−	−	−
NF0998	−	+	+	+	−	−	−	−
JR08	−	+	−	+	−	−	−	−
Mg116	−	+	−	+	−	−	+	+
RM09	−	+	−	+	−	−	+	+
SB177	−	+	−	+	−	−	+	+

[a] Hydrolytic enzyme activities and metabolite production were demonstrated by plate assays

[b] NPA, OPA, and NFb were media used to screen the inorganic phosphate dissolving bacteria, organic phosphate solubilizing bacteria, and nitrogen fixating bacteria, respectively. Here, we used the codes to stand for inorganic and organic phosphate solubilizing activities and nitrogen fixation

morphological, physiological, and biochemical properties (Table 3), and on their 16S rRNA gene sequence (Table 4). Strain JR08 was identified as a *Bacillus cereus* strain by the same method. Strain NF0919 was identified as a strain of *Streptomyces cochorusii* on the basis of its culture characteristics (Table 5), physiology, biochemical characteristics, microscopic characteristic of sporophores (Table 6), and 16S rRNA sequence (Table 4).

Toxicity test of crude extract in vitro

The crude extract showed high toxicities against mycelia of *R. solani*. Mycelial growth of *R. solani* was completely inhibited by the crude extract at a concentration of 25.0 μg ml^{-1}. In comparison, mycelial growth inhibition by Jinggangmycin (25.0 a.i. μg ml^{-1}) was 62.5%. The EC_{50} was lower with crude extract than with Jinggangmycin, revealing that the inhibitory activity of the crude extract was stronger than the positive control (Table 7).

Biological control of RSB with antifungal bacteria in the field

Rice plants were treated with foliar spray at the booting stage. In the 2013 field experiment, the percentage increase in disease severity in control plots at 7, 14, and 21 days after treatment was 15.7, 38.3, and 44.1%, respectively. The efficacy of biocontrol at 7, 14, and 21 days after treatment was 78.4, 69.5, 60.5%, respectively, in crude extract treatment plots and 77.1, 70.2, and 57.3% in fresh cells culture of SB177 treatment plots (Table 8). In comparison, the biocontrol efficacy of Jinggangmycin and Kresoxim-methyl treatment plots at 7, 14, and 21 days after treatment was 67.1, 57.9, and 48.1%; and 63.8, 51.9, and 48.5% (Table 8), respectively.

A similar trend was observed in the 2014 filed experiment and both the crude extract from NF0919 culture filtrate and fresh cells culture of SB177 showed better disease control efficacy than Jinggangmycin or Kresoxim-methyl ($P < 0.1$) (Table 9). The collective results from the 2-year field experiment suggested that the crude extract of NF0919 and fresh cells of SB177 provided better disease control efficacy than the fungicides Jinggangmycin or Kresoxim-methyl.

Discussion

Rice sheath blight is a serious disease in China [54]. Application biocontrol agent could greatly reduce the use of fungicides and thereby reduce pollution of the environment. Based on these above point of views, and looking forward to screening potential biocontrol microbial, we carried out this research.

Previous studies have indicated that cowdung (CD) microflora showing diversity, and normally contain abundant number of bacilli, lactobacilli, cocci, some identified and unidentified fungi and yeasts [37]. Ware et al. [58] reported that the lower part of the gut of the cow contains various microorganisms including *Lactobacillus plantarum*, *L. casei*, *L. acidophilus*, *B. subtilis*, *Enterococcus diacetylactis*. Other than these, the rumen of the cow contains various species of *Bacillus* and *Bifidobacterium* and yeasts (commonly *Saccharomyces cerevisiae*) for better rumen fermentation [27]. Under normal conditions,

Table 3 Morphological and physiological characteristics of strain JR08, RM09, SB177 and Mg116

Test index	Results			
	JR08	Mg116	RM09	SB177
Gram stain	+	+	+	+
Cell shape	Rod	Rod	Rod	Rod
Cell diameter >1 μm	+	−	−	−
Formation of spores	+	+	+	+
Intumescence of spores	−	−	−	−
Circle shape of spores	−	−	−	−
Parasporal crystal	−	−	−	−
Peroxidase	+	+	+	+
Oxidase	+	+	+	+
Anaerobic growth	+	−	−	−
VP reaction	+	+	+	+
VP < pH 6	+	−	+	−
VP > pH 7	−	+	−	+
Methyl red	+	−	+	−
Glucose	+	+	+	+
Xylose	−	+	+	+
L-Arabinose	−	+	−	−
Mannitol	−	+	+	+
Lactose	−	+	+	+
Citrate	−	−	−	−
Growth at 50 °C	+	+	+	+
pH 5.7	+	+	+	+
NaCl 7%	+	+	+	+
Amylohydrolysis	+	+	+	+
Casein hydrolysis	+	+	+	+
Gelatin liquefaction	+	+	+	+
Nitrate reduction	+	+	+	+

+, positive result; −, negative result

aged CD gets invaded with several soil contaminants such as bacteria, fungi, *Trichoderma* and actinomycetes [37]. Swain and Ray [52] reported that some bacteria (isolated from CD), *B. subtilis* CM1-CM5 had the ability to inhibit the in vitro growth of fungi.

In the current study, we isolated a new *Bacillus* strain SB177 from CD microflora and identified it as a strain of *B. amyloliquefaciens*. In dual-culture assays, mycelial growth of *R. solani* was strongly inhibited by SB177. Further study revealed that the inhibited mycelia of *R. solani* cannot infect the rice leaf again (paper in preparation). Our data indicate that the control efficacy of treatment with SB177 was 77.1% in 2013 and 94.2% in 2014 after treatment 7 days (Tables 8, 9) in field test. Furthermore, studies in our experiment have shown that all these antagonistic *Bacillus* spp (*B. cereus* JR08, *B. amyloliquefaciens* Mg116, *B. amyloliquefaciens* RM09, and *B. amyloliquefaciens* SB177) were thermotolerant (up to 60 °C), which may be useful in producing commercial products.

Although the group of fluorescent pseudomonas included the species *P. fluorescens* PF1, PB2, PfALR1, pfMDU2, and *P. aureofaciens* have been reported to be the most frequently beneficial and antifungal bacteria [38–40, 47, 56], due to the characteristics of the fluorescent pseudomonas (short shelf-life of formulation), there were almost no commercial products to be used in control RSB so far.

For biocontrol applications, *Bacillus* spp. was commonly used, because they can produce endospores, and can be well compatible with commercial products in factories. Almost all of the biocontrol products of RSB were *Bacillus* spp. included the species *B. cereus*, *B. subtilis* Bs-916, *B. megaterium*, *B. subtilis* NJ-18 and *B. subtilis* MBI 600 [9, 10, 26, 30, 68]. Moreover, *B. subtilis* formulations were used to control RSB in combination with a fungicide and antibiotic (Jianggangmycin), or as single bacteria formulations [59, 60, 63].

Understanding the mechanism of control could help us improve the level and consistency of control. The modes of action reported for bacterial antagonists of fungal pathogens are antibiosis, nutrient depletion around the sites of pathogen penetration, hyper-parasitism with release of cell wall degrading enzymes, stimulation of the plant's defense capacity and induction of systemic resistance (ISR) [1, 3, 18, 29, 33, 36, 41, 45, 55].

In our study, the antagonism of selected bacterial isolates was found to involve the production of antifungal compounds. In dual-culture experiments, the wide inhibition halos produced by the bacterial isolates SB177 toward the fungal mycelia of *R. solani* suggest the release of

Table 4 Taxonomic identity of bacterial isolates obtained in this study according to 16S rRNA gene sequence

Isolate	Partial 16S rRNA gene base pairs	Accession no.	Closest GenBank library strain and accession no.	Similarity (%)
JR08	1422	HM989896	*Bacillus cereus*, GU369810.1	99
Mg116	1404	HM989897	*Bacillus amyloliquefaciens,* HM107809.1	100
NF0919	1396	HM989898	*Streptomyces cochorusii,* EF063448.1	99
RM09	1407	HM989899	*Bacillus amyloliquefaciens,* HM107806.1	99
SB177	1414	HM989900	*Bacillus amyloliquefaciens*, GU568185.1	99

Table 5 Culture characteristics of strain NF0919

Culture properties of strain NF0919	Aerial mycelium	Substrate mycelium	Soluble pigment
Gause's synthetic agar	Gray	Grape purple	–[b]
ISP-2[a]	Gray	Coffee brown	–
ISP-3	Gray	Lilac brown	Brown
ISP-5	Weak-gray white	Golden yellow	Yellow
Santa's agar	Milk white	Brown	–
Inorganic salt starch agar	Gray	Softwood yellow	–
Czapek' s agar	Cream yellow	Sailing yellow	Yellow
Glucose-asparagine agar	Gray	Yolk yellow	Yellow

[a] International *Streptomyces* project media

[b] No production

Table 6 Physiological characteristics of strain NF0919

Test item	Result	Test item	Result	Test item	Result
Glucose	+	Glycerin	+	Sodium malate	+
Mannitol	+	Lactose	+	Sodium gluconic	+
Salicin	+	Galactose	+	Sodium citrate	+
Raffinose	+	Melizitose	+	Sodium succinate	+
Starch	+	Xylose	+	Sodium malonate	−
Sorbitol	+	Erythritol	−	Sodium hippurate	−
Sorbose	−	Laetrile	−	L-Arabinose	+
Sucrose	+	Trehalose	+	Sodium tartrate	+
Melibiose	+	Ribose	+	Tyrosinase	+
Mannose	+	Cellobiose	+	Amylase	+
Maltose	+	Inositol	+	Gelatin liquefaction	+
Inulin	+	Melampyritol	−	Nitrate reduction	−
Rhammose	+	Fructose	+	Milk peptonization	+
Microscopic characteristic of sporotrichia: stiff, soft, hooked and spirality					
Microscopic characteristic of spore: ellipse and/or circle					

+, positive result; −, negative result

bacterial-diffusing antimicrobial compounds. Although our in vitro data indicate that *B. subtilis* strain SB177 inhibits *R. solani* by means of antibiosis, we have no direct evidence that antibiosis is the mode of action in the field. Given that strain SB177 provided high levels of control in the field, however, the mode of action in the field warrants investigation. Further investigations will also be aimed at identifying and characterizing such bioactive molecules.

Bioactive compounds, because of their natural origin, are biodegradable and do not leave toxic residues or byproducts to contaminate the environment, whereas commercial fungicides pose severe toxicity to humans, plants and animals [34]. There were lots of reports about the use of *Streptomyces* spp. to control RSB [2, 6, 21, 31, 43, 44, 50, 62, 65]. From the beginning of 1970s, China and Japan were all used the metabolic products validamycin (purificated from the metabolites of *S. hygroscopicus* var. *limoneus* nov. var. in Japan and *S. hygroscopicus* var. *jinggangensis* nov. var in China) to control RSB [8, 21]. Some reports have clarified that the efficacy of validamycin (also named jinggangmycin in China) to RSB was decreased [23, 61, 66]. Therefore, screening and discovery of potential candidate isolates of actinomycetes appears to be of great significance.

In our present study, cowdung-associated *Streptomyces* species found was not previously reported as *R. solani* antagonists. It was a new isolate of *Streptomyces* spp., labeled strain NF0919, was selected on the basis of its antifungal activity to RSB in vivo and in vitro conditions. The strain was identified as a strain of *S. cochorusii*. A 2-year field experiment revealed that 167.0 mg a.i. l^{-1} of the crude extract from NF0919 culture filtrate effectively reduced the development of RSB from 78.4 to 98.1%. The biocontrol efficacy was higher than the 63.1–79.7% efficacy of 300.0 mg a.i. l^{-1} of the fungicide Kresoxim-methyl and the 67.1–83.8% efficacy of 500.0 mg a.i. l^{-1} of

Table 7 Inhibition effects of crude extract of culture filtrate produced by *S. cochorusii* strain NF0919 against mycelial growth of *R. solani* in vitro

Antifungal samples	Concentrations (μg ml^{-1})							Regression equation	r	EC_{50} (μg ml^{-1})	95% Confidence limit (μg ml^{-1})
	50.0 Inhibitory rate (%)	25.0	12.5	6.25	3.125	1.5625	0.78125				
Crude extract	100.00	100.00	91.25 ± 0.41	85.64 ± 0.64	69.27 ± 1.06	57.46 ± 2.49	36.46 ± 2.85	$y = 4.8494 + 1.4222x$	0.99	1.28	1.10–1.48
Jinggangmycin	74.15 ± 0.89	62.52 ± 2.39	50.44 ± 4.22	36.89 ± 2.33	24.57 ± 4.00	9.91 ± 2.69	3.81 ± 1.57	$y = 3.7791 + 1.1049x$	0.99	12.74	12.38–13.10

Table 8 Effectiveness of fresh cell culture of *B. amyloliquefaciens* strain SB177 and crude extract of culture filtrate from *S. cochorusii* strain NF0919 in suppressing the development of rice sheath blight in the field experiment after treatment 7, 14, and 21 days (2013)

Treatment	7 days			14 days		21 days	
	Basic disease severity[a]	Increase in disease severity (%)[b]	Control efficacy (%)[c]	Increase in disease severity (%)[b]	Control efficacy (%)[c]	Increase in disease severity (%)[b]	Control efficacy (%)[c]
Crude extract (167.0 mg l^{-1})	17.91 ± 2.30C	3.26 ± 0.45C	78.39 ± 6.58A	11.60 ± 2.42C	69.45 ± 7.45A	17.44 ± 2.90C	60.53 ± 15.75A
SB177 (1.0–1.2 × 10^7 CFU ml^{-1})	13.65 ± 4.38BC	3.47 ± 1.46C	77.11 ± 10.62A	11.06 ± 3.16BC	70.23 ± 11.71A	18.70 ± 4.08C	57.33 ± 10.65A
Jinggangmycin (500.0 mg l^{-1})	16.91 ± 2.74AB	5.05 ± 0.41B	67.13 ± 5.08B	15.95 ± 1.00BC	57.94 ± 6.12B	22.86 ± 0.86BC	48.05 ± 3.52AB
Kresoxim-methyl (300.0 mg l^{-1})	18.07 ± 10.16ABC	5.15 ± 3.03B	63.80 ± 26.93B	17.91 ± 5.37B	51.85 ± 18.77B	22.52 ± 4.93B	48.46 ± 13.54B
Control	17.04 ± 7.37A	15.65 ± 3.14A		38.32 ± 4.42A		44.09 ± 2.33A	

Data were subjected to one-way analysis of variance and compared using Duncan's multiple range test (DMRT) at $P < 0.01$

[a] Basic disease severity refers to the disease severity before treatments

[b] Percentage of disease severity increase = (treatment disease severity − basic disease severity) × 100

[c] Percentage of control efficacy = [(increase in disease severity of control − increase in disease severity of treatment)/increase in disease severity of control] × 100

Table 9 Effectiveness of fresh cell culture of *B. amyloliquefaciens* strain SB177 and crude extract of culture filtrate from *S. cochorusii* strain NF0919 in suppressing the development of rice sheath blight in the field experiment after treatment 7, 14, and 21 days (2014)

Treatment	7 days			14 days		21 days	
	Basic disease severity[a]	Increase in disease severity (%)[b]	Control efficacy (%)[c]	Increase in disease severity (%)[b]	Control efficacy (%)[c]	Increase in disease severity (%)[b]	Control efficacy (%)[c]
Crude extract (167.0 mg l^{-1})	0.33 ± 0.35C	0.23 ± 0.33C	98.10 ± 2.45A	2.25 ± 0.72C	88.85 ± 5.18A	9.34 ± 3.27C	69.21 ± 8.81A
SB177 (1.0 to 1.2 × 10^7 CFU ml^{-1})	0.86 ± 0.86AB	0.53 ± 0.37B	94.21 ± 4.93A	4.98 ± 0.98BC	76.38 ± 5.19B	10.16 ± 2.74C	66.32 ± 7.46A
Jinggangmycin (500.0 mg l^{-1})	1.76 ± 0.58BC	1.67 ± 0.43C	83.77 ± 1.59B	5.22 ± 0.58BC	75.17 ± 3.95B	12.86 ± 2.14B	56.92 ± 7.58B
Kresoxim-methyl (300.0 mg l^{-1})	1.00 ± 0.43ABC	2.14 ± 0.79B	79.69 ± 2.38B	6.49 ± 0.85B	68.89 ± 7.16B	16.40 ± 1.46B	44.84 ± 9.10B
Control	2.69 ± 1.20A	10.34 ± 2.58A		21.34 ± 4.05A		29.99 ± 2.61A	

Data were subjected to one-way analysis of variance and compared using Duncan's multiple range test (DMRT) at $P < 0.01$

[a] Basic disease severity refers to the disease severity before treatments

[b] Percentage of disease severity increase = (treatment disease severity − basic disease severity) × 100

[c] Percentage of control efficacy = [(increase in disease severity of control − increase in disease severity of treatment)/increase in disease severity of control] × 100

the antibiotic Jinggangmycin in controlling the disease 7 days after treatment. It was important to note that the recommended dosage of Jinggangmycin in China was only 160.0 mg a.i. l^{-1} for field control. The mechanism of Jinggangmycin to *R. solani*. is still unclear. One kind of mechanism of Jinggangmycin was the inhibition of the trehalose enzyme of *R. solani* [24]. Jinggangmycin have no direct inhibitory effect on *R. solani* in vitro test [32]. However, the metabolites of our study have direct inhibition to the mycelium of *R. solani*. As a result, it seems to conclude that the metabolic product in our study was different from the Jinggangmycin and may be a novel type of antibiotic.

Our results suggested that strains NF0919 and SB177 may be developed as a biocontrol resource for RSB control. To our knowledge, this is the first report of the use of *B. amyloliquefaciens* and *S. cochorusii* as biocontrol resources for RSB. Further research will be carried out to establish stable formulations, interpret the mechanism of the biocontrol agents, and identify the molecular structural formula of secondary metabolites.

Acknowledgements This research was supported by a Grant-in-Aid for science research from the Independent innovation Foundation (cx(12)5067), and the Science and Technological Project (NY2015019, BE2015364) of Jiangsu province. We thank the anonymous reviewers for critical comments and suggestions on the manuscript.

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