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Bacillus velezensis BM21, a potential and efficient biocontrol agent in control of corn stalk rot caused by *Fusarium graminearum*

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Abstract

The present work was conducted to screen and identify biocontrol bacteria that effectively reduce the severity of corn stalk rot (CSR) and clarify the antifungal activity of secondary metabolites. The bacterial strain (BM21) was isolated from corn rhizosphere soil that effectively reduced CSR in pot experiments. On the basis of phylogenetic reconstructions, 16S rRNA sequence analysis, and biochemical and physiological reactions, BM21 was identified as *Bacillus velezensis*. The strain exhibited remarkable antifungal activity against *Fusarium graminearum*, a pathogenic fungus that causes CSR. Extracellular antifungal substances (10%) isolated from BM21 inhibited *F. graminearum* mycelial growth by 79.2%, conidial germination by 84.0%, and conidial production by 78.1%. In addition, the extracellular antifungal substances caused mycelial malformation and ultra-structural changes. The extracellular antifungal substances were sensitive to heat and showed a degree of resistance to ultraviolet radiation. The optimum pH for antifungal activity was 6–8. In pot experiments, irrigation with aqueous extracts from BM21 (1.0 mL/plant) reduced CSR incidence by 72.4–77.4%. *B. velezensis* BM21 effectively reduced CSR incidence and showed a potential as a biocontrol agent to control CSR.

Keywords: Corn stalk rot, *Fusarium graminearum*, *Bacillus velezensis*, Biological control, Antifungal mechanism

Background

Corn (*Zea mays* L.) is an important food crop and feedstuff worldwide and provides at least 30% of the food calories to more than 4.5 billion people in 94 developing countries (Shiferaw et al. 2011; Fu et al. 2014). However, crop pathogens reduce the yield and quality of agricultural production, which could cause substantial economic losses and reduce food security at household, national, and global levels (Savary et al. 2019). Corn stalk rot (CSR) is a serious soil-borne disease caused by *Fusarium* spp., predominantly, *F. verticillioides*, *F. proliferatum*, *F. subglutinans*, *F. graminearum*, *F. incarnatum*, and *F. temperatum* (Scauflaire et al. 2011; Varela et al. 2013; Gai et al. 2016), and results in severe losses in corn production. CSR caused by *F. graminearum* is among the most devastating diseases of corn worldwide (Li et al. 2016a). At present, the selection of resistant corn cultivars, improved cultivation techniques, and seed-coating treatments are common control measures of CSR in corn production. The aforementioned *Fusarium* spp. can infect corn at any developmental stage and season. Thus, the application of most chemical fungicides as a seed coating does not effectively and continuously control CSR throughout the growing season (Li et al. 2016a).

At present, agricultural production mainly depends on chemical inputs (such as fertilizers, pesticides, and herbicides), which, all things being equal, cause a detrimental effect on the nutritional value of agricultural products and the health of farmers and consumers. Excessive use and misuse of these chemicals have caused food contamination, weed and disease resistance, and negative environmental outcomes with a serious impact on human health (Alori and Babalola 2018). However, the application of beneficial soil organisms is being considered as a sustainable and environmental friendly alternative (Jaffuel et al. 2019). Thus, biological control may overcome the shortage of chemical agents and ensure healthy growth of corn plants throughout the entire growth

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period, and therefore has become a crucial research focus.

Biological control is a promising approach in the control of plant diseases (Li et al. 2018). Plants are able to recruit protective microorganisms and enhance microbial activity to suppress pathogens in the rhizosphere (Berendsen et al. 2012). In the past decade, many studies on biological control of CSR have been undertaken, using microbes such as *Bacillus vallismortis* BV23 (Li et al. 2019), *Trichoderma asperellum* GDFS1009 (Wu et al. 2017), *Bacillus methylotrophicus* S1 (Li et al. 2016b), and *T. asperellum* ZJSX5003 (Li et al. 2016a). Among these microbes, *Bacillus* spp. are known to produce a wide array of antimicrobial compounds and inhabit diverse habitats, of which the majority of bioactive molecules are non-ribosomally synthesized peptides and lipopeptides (Fira et al. 2018).

In the present study, bacterial strains from the corn rhizosphere soil were isolated and screened in vitro for antagonistic activity against *F. graminearum*. The objectives of the study were to screen bacterial biocontrol agents for reduction of CSR severity caused by *F. graminearum*, to identify bacterial species that exhibit antagonistic activity, and to design a series of preliminary tests to evaluate the potential value of a bacterial species as a biological control agent.

Materials and methods

Isolation and screening of biocontrol bacteria

Bacterial strains were isolated from corn rhizosphere soil in Harbin, China (45° 41′ N, 126° 37′ E). The pathogenic fungus *F. graminearum* strain YJH2 (GenBank accession: MG548651), which causes corn stalk rot, was maintained in the Plant Pathology Laboratory of Northeast Agricultural University in Harbin, China. The culture media used were as follows: potato dextrose agar medium (PDA), nutrient agar medium (NA), and Luria–Bertani medium (LB). Using the standard dilution plate method for isolation of biocontrol bacteria strains, bacteria were isolated and cultured on beef extract-peptone medium plates (Li et al. 2019). The obtained bacterial strains were screened against *F. graminearum*, using the plate confrontation method (Hu et al. 2013). The tested bacterial strains were activated on the NA medium at 28 °C for 48 h and inoculated 3 cm from the center of a PDA plate (diameter 9 cm), using the parallel streak method. *F. graminearum* mycelial disks (diameter 0.7 cm) were then inoculated in the center of the PDA plates with three replicates. The PDA plates were incubated at 26 °C for 5 days. The maximum and minimum radii of *F. graminearum* colonies were measured to determine the antagonistic activity of the tested bacterial strains (Huang et al. 2017).

Antifungal spectrum

Using the described confrontation method, the antifungal spectrum of selected biocontrol bacterial strain (*B. velezensis* BM21) against 10 species of pathogenic fungi was assessed and the specific operation method is as above.

Identification of biocontrol bacteria

Tested bacterial strains were activated on the NA medium at 28 °C for 24 h. The morphological characteristics of the bacterium were observed on the NA medium. Physiological and biochemical characteristics were determined following the procedures of Schaad et al. (2001) and Dong and Cai (2001).

16S ribosomal DNA sequencing

Genomic DNA of tested biocontrol bacteria was extracted, using a DNA Extraction Kit (Kangweishiji CW Bio, Beijing, China). The 16S ribosomal RNA (rRNA) gene was amplified using the universal primers 27F (5′-AGAGTTGATC CTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTAC GACTT-3′). The 50-μL PCR mixtures contained 25 μL PCR Taq mixture (Tiangen Biotech, Beijing, China), 2 μL of 10 mM primer 1492R, 2 μL of 10 mM primer 27F, 19 μL sterile deionized water, and 2 μL DNA template. The PCR protocol consisted of initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 60 s, 58 °C for 60 s, and 72 °C for 90 s, and a final extension step at 72 °C for 10 min (Frank et al. 2008). The PCR product was detected by agarose gel electrophoresis and directly sequenced by Shanghai Biological Engineering Co., Ltd. (Shanghai, China). Based on 16S rRNA sequence data, phylogenetic trees for the identified bacterial strain were constructed using the neighbor-joining method with 1000 bootstrap replicates with MEGA 6 software (Tamura et al. 2013).

Detection of production site of antifungal active substances

A small amount of activated bacterium strain was selected with a bacteriostatic hook and inoculated into the LB medium (liquid loading 300 mL L^{-1}) at 28 °C and shaken at 170 rpm for 7 days. The suspension was then divided into two portions. One portion of the suspension was filtered through a bacterial filter (YY3014236, Millipore, USA). The second portion was ruptured using an ultrasonic cell crusher (Shanghai Hannuo Instruments Co., Ltd., Shanghai, China), then filtered through a bacterial filter. The activity of mixture extracts and extracellular antifungal substances in the filtrate was determined using a mycelial growth rate method (Li et al. 2016b). The aseptic filtrate containing the mixed and extracellular antifungal substances was added to the PDA medium to attain filtrate concentrations of 1%, 5%, and 10%. *F. graminearum* mycelial disks (diameter 0.7 cm) were inoculated in the center of PDA plates with three

replicates. The quantitative LB medium was added to PDA as the control. The PDA plates were incubated at 26 °C for 5 days. The experiment was repeated twice. The colony diameter was measured to determine the inhibitory effect on *F. graminearum*.

Effect of sterile extracellular culture supernatant on conidial germination of *F. graminearum*

Activated *F. graminearum* was inoculated on rice straw medium (dry straw 30 g, glucose 5 g, and distilled water 1 L) to induce conidial production at 26 °C for 10 days without light. Conidia were diluted to generate a conidial suspension (10^8 cfu mL^{-1}). Aseptic extracellular culture supernatant was added to conidial suspension to attain concentrations of 1, 5, and 10%. The LB medium was added to the conidial suspension as the control with three replicates. The conidial suspensions were incubated at 26 °C in an incubator. Once the percentage spore germination of the control exceeded 60%, the number of germinated conidia was scored for each treatment (100 spores per treatment). The experiment was repeated twice.

Effect of sterile extracellular culture supernatant on conidial production of *F. graminearum*

Five-day-old *F. graminearum* disks (diameter 7 mm) were transferred to rice straw medium plates and were incubated at 26 °C until the colonies had grown to 4 cm diameter. Aseptic extracellular culture supernatant was diluted to concentrations of 1, 5, and 10%, then added to the rice straw medium plates (20 mL per dish) for 20 min. The surface hyphae on the plates were scraped off, and the surrounding medium without hyphae was cut off. The excess solution on the plates was poured into a waste liquid cylinder and incubated at 26 °C for 72 h with three replicates. The LB liquid medium was added to plates as the control. The experiment was repeated twice. The concentration of the conidial suspension was measured using cell counting methods.

Effect of sterile extracellular culture supernatant on mycelial growth of *F. graminearum*

Aseptic extracellular culture supernatant was added to the PDA medium to attain concentrations of 1%, 5%, and 10%. *F. graminearum* disks (diameter 7 mm) were inoculated in the center of PDA plates with 3 replicates. An equal volume of the LB medium was added to PDA as the control. The PDA plates were incubated at 26 °C for 5 days. The experiment was repeated twice. The colony diameter was measured to determine the inhibitory effect on mycelial growth of *F. graminearum*.

Morphological and ultra-structural changes in mycelial cells induced by sterile extracellular culture supernatant

Fresh mycelia with some medium, cultured on PDA medium for 24 h, were picked up and added into sterile extracellular culture supernatant to attain concentrations of 1, 5, and 10%, then incubated at 26 °C for 24 h. Control treatments were prepared using an equal volume of the LB medium. Sample preparation for microscopic examination followed the methods of Ooi et al. (2011). The samples were observed under an optical microscope (90i, Nikon, Tokyo, Japan) and a transmission electron microscope (H-7650, Hitachi, Tokyo, Japan).

Thermal stability

Aseptic extracellular culture supernatant was treated by heating in a water bath at 20, 40, 60, 80, 100, and autoclaved at 121 °C for 20 min with untreated blank samples as the control. Using a mycelium growth rate method, the treated aseptic extracellular culture supernatant was added to the quantitative PDA medium to attain a concentration of 5%. The experiment was repeated twice. The antifungal activity of the treated broth was assessed using the same procedure described for the non-treated sterile extracellular culture supernatant.

pH stability

Using a mycelium growth rate method, aseptic extracellular culture supernatant was added to PDA media differing in pH (pH 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12) to attain a concentration of 5%. The experiment was repeated twice. The antifungal activity of the treated broth was assessed using the same procedure described for the non-treated sterile extracellular culture supernatant.

Ultraviolet stability

Aseptic extracellular culture supernatant was irradiated for 10, 20, 30, or 60 min within a distance of 1 m from an ultraviolet lamp (100 μW cm^{-2}), respectively. Using a mycelium growth rate method, treated aseptic culture supernatant was added to the quantitative PDA medium to attain a concentration of 5%. The experiment was repeated twice. The antifungal activity of the treated broth was assessed, using the same procedure described for the non-treated sterile extracellular culture supernatant.

Application of *Bacillus velezensis* strain BM21

Lipopeptide-producing fermentation medium contains sucrose 200 g, NH_4NO_3 2 g, KH_2PO_4 3 g, Na_2HPO_4 10 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, yeast extract 0.2 g, $CaCl_2$ 0.7 μg, $MnSO_4{\cdot}4H_2O$ 1 μg, and distilled water 1 L.

Seed liquid of *B. velezensis* strain BM21 activated for 24 h was added to the lipopeptide-producing

Table 1 Inhibitory spectrum of *Bacillus velezensis* BM21 against 10 species of pathogenic fungi

Strains	Maximum/minimum radius (SE)	Strains	Maximum/minimum radius (SE)
Athelia rolfsii	1.8 ± 0.1efg	*Fusarium oxysporum*	2.1 ± 0.1de
Phytophthora megasperma	1.9 ± 0.2e	*Cladosporium allii*	1.7 ± 0.3de
Alternaria alternate	1.8 ± 0.1ef	*Pythium graminicola*	2.2 ± 0.1c
Fusarium verticillioides	2.0 ± 0.1e	*Rhizoctonia solani*	2.0 ± 0.1e
Thanatephorus cucumeris	2.8 ± 0.3b	*Typhula incarnata*	2.6 ± 0.3bc

The above data was the average of three times. Results were expressed as better (maximum/minimum radius > 2); means followed by a similar letter are not significantly different at 0.05 level of probability, Duncan's multiple range test

fermentation medium (liquid loading 300 mL L^{-1}) with 5% inoculum and incubated at 28 °C for 48 h at 180 rpm. The pH value of the BM21 fermentation medium was then adjusted to 8.0 using 0.1 M NaOH, and the bacteria were removed by centrifugation for 10 min at 12000 r/min. The pH value of the sterile supernatant was adjusted to 2.0 with 0.1 M HCl and refrigerated overnight at 4 °C for completion of precipitation. The precipitate was collected and washed twice with HCl at pH 2.0 and extracted five times with methanol.

The lipopeptide extract was obtained by decompression drying of the methanol extract solution in a rotary evaporator (RE-52AA, Shanghai Ya Rong Biochemical Instrument Co. Ltd., Shanghai, China) at 35 °C. The lipopeptide extract was diluted to saturation with methanol for storage. After soaking 200 g sorghum grains in sterile water for 12 h, the sorghum grains were boiled for 30 min, transferred to flasks (50 g 250 mL^{-1}), and treated twice with damp-heat sterilization at 121 °C for 30 min. Five activated *F. graminearum* disks (diameter 0.7 cm) were transferred to flasks containing aseptic sorghum grains and incubated at 26 °C for 5–6 days with manual shaking once per day. The experiment was repeated twice.

Table 2 Biological and biochemical analysis of antagonistic bacteria BM21

Characteristic	Results	Characteristic	Results
Gram strain	+	Lactose	+
Catalase	+	Sucrose	+
Casein	+	Mannose	+
Aerobic	+	Glucose	+
Mannose	+	Fructose	+
V.P. test	+	Arabinose	+
Gelatin liquefaction	+	Xylose	−
Nitrate reduction	−	Mannitol	+
M.R. test	+	Phaseomannite	−
Starch hydrolysis	+	Rhamnose	−
Hydrogen sulfide	−	Cellobiose	+

"+" indicates positive response; "−" indicates negative response

In sequence, aseptic soil (peat soil: vermiculite, 2:1, v/v), of which the organic matter was 25.6 g/kg, available nitrogen 148.5 mg/kg, available phosphorous 50.6 mg/kg, rapidly available K 208.4 mg/kg with pH value 7.3, was placed in a pot (diameter 15 cm and 10 cm high), and then 20 sorghum grains inoculated with *F. graminearum* were placed on the soil surface, followed by 0.5 cm soil, five seeds, and finally 1.5 cm soil. Then, NPK (15-15-15) compound fertilizer (fertilizer:water for 1:20) was used to irrigate the root once every 2 weeks. Saturated crude lipopeptide extract of BM21 diluted 4000 times with tap water was used to irrigate each pot at time 0 h and again after 7 days with three replicates. An equal volume of sterile water was applied in the control. The seedlings were incubated in a greenhouse at 23 °C ± 5 °C with a photoperiod of 12 h/12 h (light/dark) for 20 days. The experiment was repeated twice. The specific treatments applied were as follows: (1) 0.2 mL lipopeptide extract applied as a root drench to each seedling after emergence; (2) 0.6 mL lipopeptide extract applied as a root drench to each seedling; (3) 1 mL lipopeptide extract applied as a root drench to each seedling; (4) 2 mL 64% Mancozeb WP (5×10^{-3} g $plant^{-1}$) applied as a root drench to each plant; and (5) non-treated control. The pots were placed in a greenhouse at 25 ± 3 °C. The seedlings were watered daily using overhead irrigation to maintain soil moisture. Disease incidence was evaluated 20 days after sowing and was quantified as the percentage of diseased plants (Li et al. 2018). Disease reduction (%) was calculated as

$$\frac{\text{disease incidence of untreated control} - \text{disease incidence of the treatment}}{\text{disease incidence of the untreated control}} \times 100\%$$

Data analysis

All experiments were conducted twice under similar conditions. Data were analyzed by analysis of variance (ANOVA), using IBM SPSS Statistics 19.0 (IBM Corporation, Armonk, NY, USA). Significant differences among treatment means were distinguished using Duncan's multiple range test ($P < 0.05$).

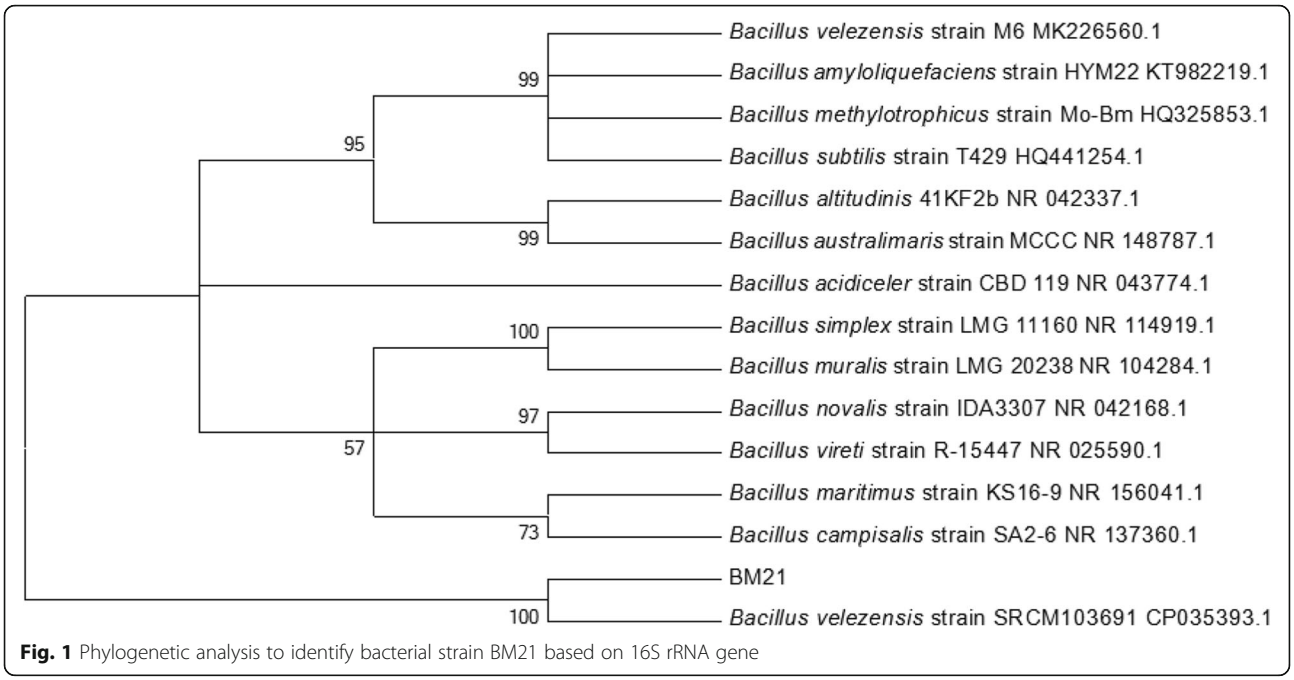

Fig. 1 Phylogenetic analysis to identify bacterial strain BM21 based on 16S rRNA gene

Results and discussion

Screening of biocontrol bacteria

F. graminearum was the target pathogen in the present study. A total of 158 bacterial isolates were screened. Three isolates showed strong antagonistic activity against *F. graminearum*, using a dual-culture method. The isolate BM21 exhibited the strongest antifungal activity, with the longest radius/shortest radius ratio of 3.2.

Antifungal spectrum of *B. velezensis* BM21

The isolate BM21 showed an inhibitory effect on mycelial growth of 10 species of plant-pathogenic fungi (Table 1). Among these fungi, a strong inhibitory effect (maximum radius: minimum radius > 2) was exerted on six fungi, namely, *F. verticillioides*, *Thanatephorus cucumeris*, *Typhula incarnata*, *F. oxysporum*, *Pythium graminicola*, and *Rhizoctonia solani.*

Many *Bacillus* strains show broad-spectrum antimicrobial activity and are widely used in the biocontrol of crop diseases (Luo et al. 2015). *Bacillus velezensis* has been formulated into the commercially available fungicide Botrybel (Agricaldes, Spain) owing to its activity against *Botrytis cinerea* (Romanazzi and Feliziani 2014). But, *B. velezensis* used to control CSR had not been reported.

Identification of bacterial strain BM21

Strain BM21 colonies were subspherical, milk-white, creamy, semitransparent, surface folded, with a crater-like depression. The cells were a short rod, aerobic, Gram-positive, and the spore was oval. Strain BM21 utilized arabinose, glucose, lactose, cellobiose, sucrose, mannose, fructose, and mannitol, but did not utilize rhamnose, xylose, and phaseomannite. The isolate produced catalase, caseinase, gelatinase, and amylase, but did not produce hydrogen sulfide and nitrate reductase (Table 2). In addition, the V-P test and M-R test were positive.

Genomic DNA of BM21 was extracted, and the 16S rRNA gene was amplified and sequenced using the

Table 3 Detection of production position of antifungi substances from BM21 against *Fusarium graminearum*

Position	Application dose	Colony diameter[b]	Inhibition rate (%)
Mixed substances[a]	1%	4.3 ± 0.3b	45.6
	5%	4.0 ± 0.2b	49.4
	10%	2.0 ± 0.1c	74.7
Extracellular	1%	4.6 ± 0.5b	41.8
	5%	3.3 ± 0.2b	58.2
	10%	1.9 ± 0.3c	75.9
	CK	7.9 ± 0.2a	

[a]Mixed intracellular and extracellular substances; [b]Mean ± standard error of *F. graminearum* colony diameter in antagonistic test with BM21. Values within the column followed by different letters are significantly different according to Duncan's multiple range tests ($P < 0.05$)

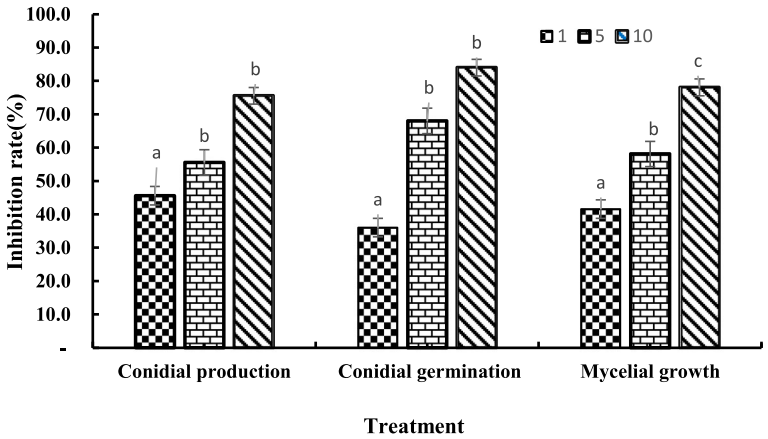

Fig. 2 Effect of extracellular culture supernatant of BM21 on mycelial growth, conidial germination, and conidial production of *Fusarium graminearum*

universal primers 27F and 1492R. The 16S rRNA sequence (GenBank accession no. MK791317) was 1445 bp in length and showed 100% homology with *Bacillus velezensis* strain M6 (GenBank accession no. MK226560.1). Phylogenetic analysis of sequence data for the 16S rRNA gene revealed that BM21 and *B. velezensis* belonged to the same evolutionary lineage within the *Bacillus* clade (Fig. 1). On the basis of the phylogenetic analysis, 16S rRNA sequence analysis, biochemical and physiological reactions, and morphological characteristics, the strain BM21 was identified as *B. velezensis.*

Among biocontrol microbes, *Bacillus* spp. exert their biocontrol capability predominantly through inhibitory activity on the growth of plant pathogens, as well as inducing systemic resistance in plants and competing for ecological niches with plant pathogens (Fira et al. 2018). Among *Bacillus* spp., *B. velezensis* is an aerobic, Gram-positive, endospore-forming bacterium that promotes plant growth (Rabbee et al. 2019). *Bacillus* spp. have

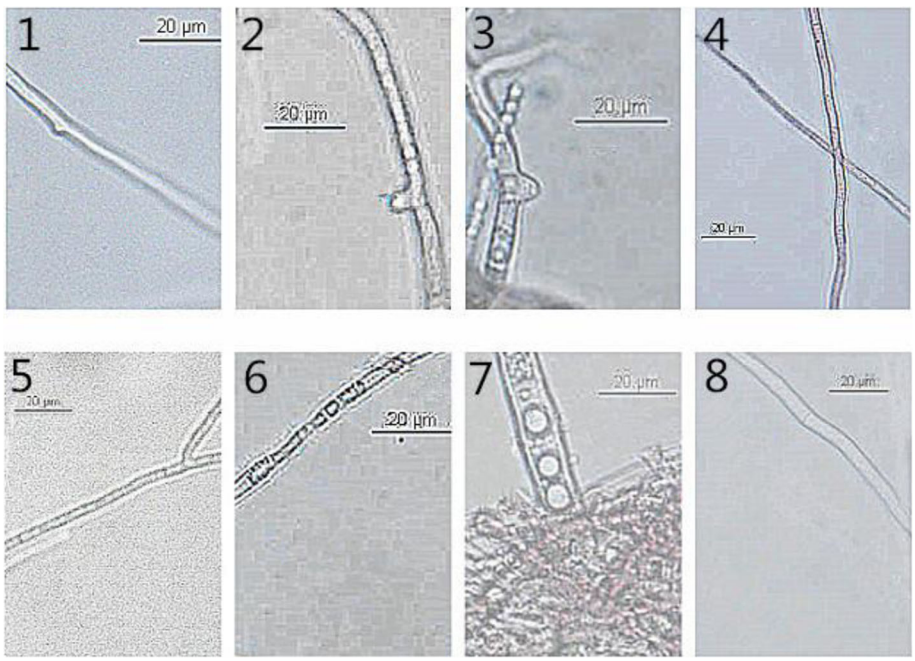

Fig. 3 Effect of extracellular culture supernatant of BM21 on mycelium of *F. graminearum* (magnified × 400). **4** and **8** were non-treated control; **1** and **5**, **2** and **6**, and **3** and **7** indicate coarser hyphae and cytoplasmic granulation becoming more and more serious with the increase of concentration of extracellular culture supernatant (1%, 5%, and 10%), respectively

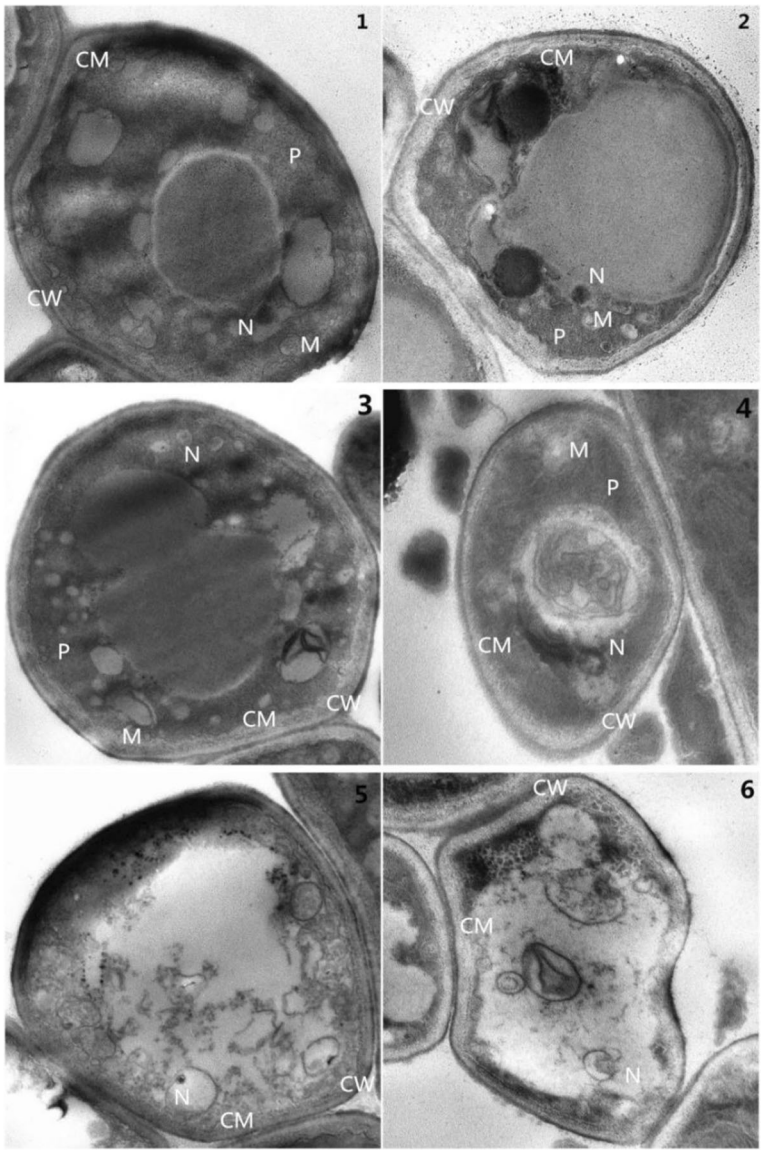

Fig. 4 Effect of extracellular culture supernatant of BM21on ultrastructure of *F. graminearum* hyphae (magnified × 30,000 times). **1** was non-treated control; **2**, **3**, and **4** indicate after treatment with 1% extracellular culture supernatant; **3** and **4** indicate after treatment with 5% extracellular culture supernatant; **5** and **6** indicate after treatment with 10% extracellular culture supernatant. CW stands for cell wall; CM stands for cytoplasma membrane; M stands for mitochondria; N stands for nucleus; P stands for ribonucleoprotein particle; V stands for vacuole

been the most frequently exploited bacteria for commercial development of biocontrol agents owing to their ability to form endospores, which can survive heat exposure and desiccation, and their capacity to be formulated into stable dry powders with a long shelf life (Guetsky et al. 2002; Chowdhury et al. 2013). *Bacillus* spp. show the potential to produce more than 45 antimicrobial molecules (Urdaci and Pinchuk 2004; Stein 2005). Three families of cyclic lipopeptides produced by *Bacillus* spp., namely, surfactins, iturins, and fengycins, exhibit antimicrobial activity and the majority of the mechanisms that account for the biocontrol effects of different *Bacillus* strains have been well documented (Romero et al. 2007; Zeriouh et al. 2014). Thus, the present research focused on identifying *Bacillus* spp. as biocontrol agents to reduce CSR severity caused by *F. graminearum*. In the present study, a strain identified as *Bacillus velezensis*, which was named BM21, exhibited remarkable inhibitory activity against *F. graminearum*.

Production site of antifungal substances

The antifungal activity of mixed and extracellular culture supernatant was determined using a mycelial growth rate method. The 10% concentration of extracellular

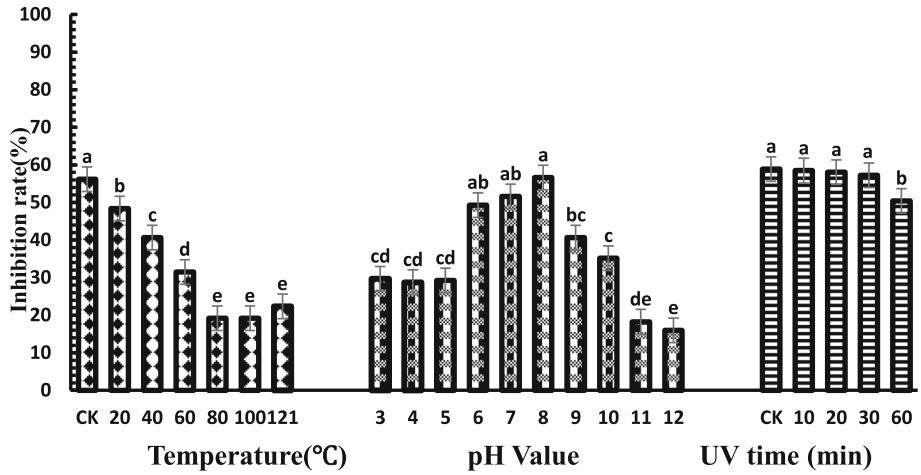

Fig. 5 Effect of temperature, pH value, and time on extracellular culture supernatant from BM21against *Fusarium graminearum*

culture supernatant had the strongest inhibitory effect on mycelial growth of *F. graminearum*, which decreased the mycelial growth rate by 75.9% relative to the control (Table 3). Non-significant difference in inhibitory effect between mixed and extracellular culture supernatant at the corresponding concentration was observed. It was concluded that the main antifungal substances of strain BM21 were contained in the extracellular culture supernatant.

Effect of BM21 extracellular culture supernatant on mycelial growth, conidial germination, and conidial production of *F. graminearum*

Treatment with different concentrations of extracellular culture supernatant isolated from BM21 had inhibitory effects on mycelial growth and conidial germination and production of *F. graminearum*. The extracellular culture supernatant (10%) inhibited mycelial growth by 79.2%, conidial germination by 84.0%, and conidial production by 78.1% (Fig. 2).

Effects of BM21 on mycelial morphology and ultrastructure

Microscopic examination of mycelia of *F. graminearum* treated with BM21 culture supernatant showed that coarser hyphae and cytoplasmic granulation were increasingly severe with an increase in the concentration of the extracellular culture supernatant (from 1 to 10%) (Fig. 3). In contrast, the hyphae of the control cultures were fine and uniform, with a smooth surface, dispersed protoplasm, and uniform growth point.

Treatment of *F. graminearum* with 1% extracellular culture supernatant of BM21 increased the volume and number of mitochondria (Fig. 4). Nuclear pyknosis, mitochondrial swelling, vacuolization, and thickening of the cell wall on the upper hyphal surface were observed in hyphae of colonies treated with 5% culture supernatant. Cytoplasmic necrosis and disintegration of organelles were observed in response to treatment with 10% culture supernatant.

Table 4 Lipopeptide extracts of BM21 for the control of *Fusarium graminearum*

Experiment no.	Treatment	Application dose	Emergence rate (%)	Disease index (%)	Disease reduction (%)
1	Lipopeptide extracts	0.2 mL/plant	93.3	17.6 ± 0.4b	58.4
		0.6 mL/plant	100.0	14.1 ± 0.3b	66.7
		1.0 mL/plant	86.7	11.7 ± 0.4c	72.4
	64% Mancozeb WP	5×10^{-3} g/plant	86.7	16.5 ± 0.3b	61.0
	CK		100.0	42.2 ± 0.4a	
2	Lipopeptide extracts	0.2 mL/plant	86.7	14.4 ± 0.1b	60.0
		0.6 mL/plant	86.7	13.0 ± 0.2b	64.1
		1.0 mL/plant	93.3	8.2 ± 0.3c	77.4
	64% Mancozeb WP	5×10^{-3} g/plant	86.7	11.3 ± 0.3bc	68.7
	CK		80.0	36.11 ± 0.3a	

Assessment of the antagonistic activity against *F. graminearum* of extracellular culture supernatant isolated from BM21 showed that mycelial growth, conidial germination, and conidial production were effectively inhibited. In addition, the extracellular culture supernatant caused mycelial malformation and ultra-structural changes. Similar results were obtained for extracellular culture supernatant isolated from *B. methylotrophicus* against *F. graminearum* (Li et al. 2016b) and from *Bacillus vallismortis* against *F. graminearum* (Li et al. 2019).

The present results also indicate that BM21 can play an important role in prevention and control for both infection and reinfection by *F. graminearum*. These qualities may be a prerequisite for effective control in the field. In a future experiment, we aim to identify active substances that inhibit infection and reinfection by *F. graminearum.*

Stability of antifungal active substances

The active extracellular culture supernatant showed a high sensitivity to heat (Fig. 5). With the increase in temperature, the inhibitory effect decreased rapidly. The stability of the extracellular culture supernatant was optimal at pH 6–8 (Fig. 5). The substances showed a degree of stability under ultraviolet irradiation, with the inhibitory effect remaining unchanged within 30 min after irradiation (Fig. 5).

Bacillus velezensis has been formulated into the commercially available fungicide Botrybel (Agricaldes, Spain) owing to its activity against *Botrytis cinerea* (Romanazzi and Feliziani 2014). The present results are generally consistent with active antifungal substances isolated from *B. subtilis* HAINUP40 (Wu et al. 2019). On the basis of the abovementioned results, we speculate that the active extracellular culture supernatant of BM21 is complex lipopeptides.

Application of BM21 lipopeptide extract

Saturated lipopeptide extracts of BM21 diluted 4000 times were used to irrigate corn seedlings (Table 4). Treatment with each concentration of the lipopeptide extract was effective in controlling the incidence of CSR than the control. The antifungal activity of each lipopeptide extract treatment and of a chemical fungicide (64% Mancozeb WP) was extremely similar. Among the treatments, the application of 1.0 mL lipopeptide extract per plant was the most effective in controlling CSR, with 72.4 and 77.4% disease reduction observed in both trials.

The effectiveness of *B. velezensis* in the control of CSR caused by *F. graminearum* has not been reported previously. The strain BM21 showed a strong antifungal activity and effective control with favorable prospects for its practical application. Further studies will focus on the development, practical use of formulations, and analysis of the active components of *B. velezensis* BM21.

Conclusion

On the basis of phylogenetic reconstructions, 16S rRNA sequence analysis, and biochemical and physiological reactions, BM21 was identified as *Bacillus velezensis* that exhibited a remarkable antifungal activity against *F. graminearum* causing CSR. In addition, the antifungal substances caused mycelial malformation and ultra-structural changes. The active antifungal substances were sensitive to heat and showed a degree of resistance to ultraviolet radiation.

Abbreviations

ANOVA: Analysis of variance; CSR: Corn stalk rot; LB: Luria–Bertani medium; NA: Nutrient agar medium; PDA: Potato dextrose agar medium

Authors' contributions

WS, SL, and YGL designed the experiment. WS, WZ, and FQC conducted the experiment and wrote the article. XYH helped in the statistical analysis. JY B and YGL revised the article. All authors approved the final article after reading.

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Availability of data and materials

The data used and analyzed during this project are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

N/A

Consent for publication

N/A

Competing interests

The authors declare that they have no competing interests.

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