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Biocontrol potential and mode of action of entomopathogenic bacteria *Xenorhabdus budapestensis* C72 against *Bipolaris maydis*

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HIGHLIGHTS

- *Xenorhabdus species* have the potential as a novel biocontrol bacteria resource.
- *X. budapestensis* C72 CFCM has broad spectrum inhibitory effect on fungal pathogens.
- C72 CFCM reduce the colonization of *Bipolaris Maydis* on the leaf surface of maize.

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ABSTRACT

Southern corn leaf blight (SCLB) caused by *Bipolaris maydis* is an important foliar disease of maize. In this study, a nematode-symbiotic bacterium *Xenorhabdus budapestensis* strain C72 was identified with remarkable inhibiting effect on mycelial growth and spore germination of *B. maydis*. The *in vitro* assay revealed that C72 cell-free culture media (CFCM) with thermostability exhibited broad-spectrum antifungal activities against other several important plant pathogenic fungi. The early colonization of *B. maydis* were significantly impaired by CFCM treatment under phytotron condition. This antagonism is likely to be the main contributor to the highly efficient plant protection of 40% (v/v) CFCM treatment against *B. maydis*, and the relative control effect reached to 59.15% and 77.96% in greenhouse and field experiments, which was comparable to the effect of fungicides. Moreover, we found that extracellular enzymes secreted by symbiotic bacterium may be one of the reasons for the antifungal potential of C72. Beside direct antagonistic effects provided by the bacterium, defense related genes were induced in maize after CFCM treatment. In summary, this study reported the first systematic evaluation of the effect of *X. budapestensis* C72 in controlling SCLB and exploration of its mode of action, then indicating that entomopathogenic bacteria have the potential to become a new and efficient biological control resource for plant fungal disease management.

1. Introduction

Maize is the largest grain crop in China (Wang et al., 2014), for food, and it is also an important feed crop and as main indispensable raw material for the energy industry and the pharmaceutical industry in the world (Ranum et al., 2014). Therefore, the sustainable and safe production of maize has a great impact on the development of the whole national economy. Southern corn leaf blight (SCLB) caused by filamentous fungus *B. maydis* is one of the most important diseases in China. It can occur throughout the growing season and its symptoms appear as lesions on the leaf blade and ear husk (Yoder, 1988). SCLB usually reduces the production of maize considerably by 10% to 46% in epidemic years (Dai et al., 2020). The most profound and harsh lesson that Mother Nature inflicted on agronomists and North American maize industry was the SCLB epidemic in 1970 and 1971, when the epidemy destroyed 15% of crops with an estimated loss of 1.0 billion USD (Tatum, 1971). In recent years, the promotion of tillage cultivation techniques such as no-tillage continuous cropping and fieldplot straw returning in China has

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increased the accumulation of plant fungal pathogens leading likely to SCLB increasing incidence. Therefore, it is necessary to strengthen the prevention and control of SCLB.

Traditionally, application of synthetic chemical fungicides is the major practice for controlling SCLB (Dai et al., 2018). However, the use of chemicals has been increasingly restricted due to public concerns about the negative impacts of fungicide residues on human health and environment (Eljounaidi et al., 2016). Furthermore, an increased level of resistance to fludioxonil, carbendazim and thiophanate-methyl had already appeared in *B. maydis* due to repeated and abused use of chemical pesticides (Dai et al., 2018; Han et al., 2017). Hence, it is necessary to develop an effective and environment-friendly alternative or supplement methods for controlling this disease. Due to its high potential as alternative/complement to pesticides, biological disease control is now generally recognized and constitutes an attractive tool in integrated pest management (Raymaekers et al., 2020). A previous study found that the application of a plant growth-promoting rhizobacterium (PGPR) *Bacillus cereus* C1L in the maize rhizosphere effectively protected maize from SCLB under greenhouse and field conditions (Huang et al., 2010). Similarly, *B. amyloliquefaciens* B9601-Y2 reduced SCLB severity in treated maize plants with 61.38% decrease in disease index (Cui et al., 2019). Nevertheless, most of the biocontrol bacteria reported to reduce SCLB are *Bacillus* species, and the reports of other species against *B. maydis* are relatively limited. It is necessary to explore more genera of biocontrol bacteria resources in order to screen new antagonistic microbial candidates for controlling the SCLB epidemic.

The genus *Xenorhabdus* of the family *Enterobacteriaceae*, known as entomopathogenic bacteria, is symbiotically associated with nematodes of the genus *Steinernema* (Dreyer et al., 2018). Consistently, nematode–bacterium symbiont has been used to control agricultural underground pests (Chitra et al., 2017). It is worth noting that extensive studies have proved that the effectiveness of the symbiont is mainly attributed to the bacteria partners, which are released into the insect hemocoel to kill the host insects, Bacteria partners produced bioactive compounds, including lytic enzymes, toxic proteins, immunosuppressors and antibiotics. These active compounds are involved in pathogenicity of symbiont to host insects. Also, these compounds, especially the secondary metabolites, have excellent antagonistic activity in protecting the insect cadaver against food competitors from soil living organisms like bacteria, fungi, and other nematodes (Dreyer et al., 2018; Shi and Bode, 2018). Regrettably, the main attention of scientists has not been attracted to expanding the application of symbiotic bacteria alone as multipurpose tools in agriculture besides being insecticide. To our knowledge, few studies have been designed for evaluating symbiotic bacteria from nematodes in biological control of plant diseases (Vozik et al., 2015). Not surprisingly, there is no report to date about using *Xenorhabdus* species as biocontrol agents to control SCLB. It is then reasonable to hypothesize that the effectiveness of the bioactive compounds produced by symbiotic bacteria against bacteria and fungi would allow growers to effectively control SCLB in an eco-friendly way.

The main objective of this study was to select and identify a biocontrol agent candidate from 29 entomopathogenic bacteria for controlling SCLB. One purpose was to evaluate the control efficiency of selected entomopathogenic bacteria on suppressing SCLB in the greenhouse and field trials. Another was to clarify the preliminary mechanism of action of cell-free culture media (CFCM) on inhibiting the mycelial growth and spore germination of *B. maydis*. The present study provided insight into the biocontrol activity of entomopathogenic bacteria on plant fungal pathogens and experimental basis for the development and application of entomopathogenic bacteria as biocontrol agents for plant disease control.

2. Material and methods

2.1. Test strains and dual culture assay

The present study was conducted with 29 strains of entomopathogenic bacteria, which were preserved in the State Key Laboratory of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences. All bacterial strains were purified by re-streaking on Nutrient Brome Thymol-blue Agar (NBTA) indicator plates containing appropriate antibiotics (ampicillin at 100 $\mu g \cdot mL^{-1}$) at 28 ℃. The NBTA plates were supplemented with bromothymol blue (0.025%, w/v) and 2,3,5-triphenyltetrazolium (0.004%, w/v), and it was used to identify primary phase cell. A characteristic feature of symbiotic bacteria is the phenomenon of phase variation, the two phases of which are the primary and the secondary phase. The primary phase cells produce bioactive compounds, while secondary phase cells do not elaborate products normally associated with the primary phase cell type (Ehlers, 2001).

The antagonistic efficacy of isolates against *B. maydis* was preliminary assessed by using the dual culture method (Abdel-Rahim and Abo-Elyousr, 2017). A 5-mm diameter *B. maydis* mycelial plug from the edge of a 5-day old colony was placed on the center of a potato dextrose agar (PDA) plate. Then, 5 μL of bacterial suspension was streaked on parallel line about 3.0 cm away from both sides of plug. Negative control plates were inoculated with 5 μL of sterilized LB broth instead of suspension. Plates were incubated at 28℃ until the mycelial growth in control cover the entire surface of the plate. The experiment was repeated four times. The strain showing the strongest antifungal effect was selected for further studies.

2.2. Phenotypic and molecular phylogenetic analysis

Biochemical and cultural characteristics of selected biocontrol bacterium C72 was determined following described previously by Glaeser et al. (2017). Briefly, the reaction to Gram staining, motility, oxidase and catalase activities were tested. To identify strain C72, Genomic DNA of bacterial isolates was extracted using EasyPure Bacteria Genomic DNA Kit (TransGEN, Beijing, China). Universal primer sets (27F and 1492R; gyrB-F and gyrB-R) were used to amplify the 16S ribosomal RNA (*16S rRNA*) gene and the DNA gyrase subunit B (*gyrB*) gene, respectively. The reaction condition of amplification was as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 90 s, and a final extension at 72 °C for 10 min. The PCR amplicons were sequenced by Shanghai Sangon Biological Technology and Services Co., Ltd (Shanghai, China). DNAMAN version 5.2.2 was used to modify and produce consensus sequences which were identified by basic local alignment search tool (BLAST) against the NCBI nucleotide database. The *16S rRNA* and *gyrB* sequences obtained in this study had been uploaded to GenBank under nucleotide sequence accession number MT967912 and MW020592. Phylogenetic and molecular evolutionary analyses were conducted based on *16S rRNA* and *gyrB* gene sequences using the Neighbor-Joining (NJ) method with Kimura's two-parameter model in MEGA X (Kumar et al., 2018). Bootstrap test was conducted with 1000 replicates.

2.3. In vitro tests of broad-spectrum antimicrobial activities of C72 CFCM

For preparing bacterial suspension and CFCM of strain C72, 2 mL log phase cell suspension was re-inoculated into 1000 mL Erlenmeyer flasks containing 200 mL of sterile LB and was cultivated at 28℃ on a laboratory shaker at 200 rpm for 72 h. The cells were removed by centrifugation (12,000 rpm, 25℃, 10 min) and the supernatant was filtered through a 0.22 μm pore size filter (Merck Millipore, Darmstadt, Germany) to obtain the CFCM. The antimicrobial activities of bacterial suspension and CFCM of C72 against 17 pathogenic fungi, these pathogenic isolates from the genera *Valsa ceratosperma*, *Bipolaris sorokiniana*,

B. zeicola, *Botryosphaeria dothidea*, *Rhizoctonia solani*, *Phytophthora capsici*, *Alternaria solani*, *A. alternata, Curvularia lunata*, *Verticillium dahliae*, *Botrytis cinerea*, *Exserohilum turcium*, *Fusarium oxysporum*, *F. graminearum*, *F. pseudograminearum*, *F. culmorum* and *Magnaporthe grisea,* was respectively tested using dual culture method and "poisoned" PDA plate method as described above. All experiments were repeated six times. In "poisoned" PDA plate assay, we compared the inhibition effect of C72 CFCM on mycelial growth of tested fungi at 0, 4 and 40% (v/v) dilutions.

2.4. Control effect of C72 CFCM on SCLB under greenhouse conditions

Maize seedlings (cv. 'Xianyu-335') were sown in an 18-cm-diameter pot, five plants per pot. All pots were maintained in greenhouse for 3–4 weeks at 25℃ under 14 h photoperiod of fluorescent light and 10 h of darkness. At 4–5-true leaf stage, bacterial suspension, cell pellets and CFCM of C72 at 2.5%, 10%, and 40% (v/v) dilutions were respectively sprayed on the leaves until it running off from the leaves. To prepare the initial sample of cell pellets, the supernatant was discarded by centrifugation (6,000 rpm, 25℃, 10 min) and resuspended in an equal volume of sterile water. Sterile water was used as the negative control and 400 $mg \cdot mL^{-1}$ carbendazim (CAS No.:1897-45-6) and 500 $mg \cdot mL^{-1}$ chlorothalonil (CAS No.:10605-21-7) were used as the positive control. One day after application, the spore suspension of *B. maydis* (1×10^5 spores mL^{-1}) was sprayed on leaves; the plants were then moistened with a humidifier at 35℃ for 24 h. The experiment was performed in three replicates per treatment, 10 seedlings per replicate. The disease severity was investigated on day 14 after *B. maydis* inoculation according to a rating scale of 1, 3, 5, 7, 9 scales (no symptoms, 1–10%, 11–25%, 26–50%, 51–75%, and 76–100% leaf area covered with speck), and the disease index and relative control effect were calculated. Disease index (DI) (%) = ∑(number of affected leaves × corresponding disease severity)/(9 × number of all investigated leaves) × 100.

2.5. Control effect of C72 CFCM on SCLB under field conditions

Field trials were conducted at the experimental farm of Baoding city (38°58′N, 115° 27′E, 24 m elevation) Hebei province during the growth season (from June to October) of 2019 and 2020. The annual temperature is approximately 13.4℃ and the annual precipitation is approximately 550 mm. Maize seeds (cv. 'Xianyu-335′) were sown at 30 cm intervals and 50 cm inter-row spacing and grow naturally for seven to eight weeks. Within the first field trial, five treatments including 10% and 40% CFCM, 400 $mg \cdot L^{-1}$ carbendazim (CAS No.:1897-45-6), 500 $mg \cdot L^{-1}$ chlorothalonil (CAS No.:10605-21-7) and sterile water (negative control) were sprayed one day before inoculation of the *B. maydis* spore suspension (1×10^5 spores mL^{-1}) until running off from leaves. Each treatment consisted of three replicates and each replicate contained 80 plants. Disease severity was investigated for all plants in each plot at 14 and 21 days after inoculation. The disease index and relative control effect were calculated. A field trial with same procedures was also repeated in 2020. All other treatments were same as those in the first trial, except that only one fungicide (500 $mg \cdot L^{-1}$ chlorothalonil) was tested, the data was collected at 14 days after inoculation. Each treatment contained 120 plants in triplicate.

2.6. Observation on colonization of Bipolaris maydis on maize leaves

A *B. maydis* strain harbouring plasmid pCAMBIA2301-eGFP with green fluorescence protein gene and hygromycin B phosphotransferase gene (*hygB*) was a gifted from Prof. Kong (Hebei Academy of Agricultural and Forestry Sciences) and was used for colonization studies. Five-day old PDA plate cultures of e-GFP tagged *B. maydis* were flooded with 10 mL sterile distilled water and carefully scraped with a paint brush to harvest spores. The spore suspension was filtered through four layers of sterilized cheesecloth, and the concentration was adjusted to 1×10^5 spores mL^{-1} using a hemocytometer under microscope. Maize seedlings were prepared in the same way as the greenhouse experiment. At 4–5-true leaf stage, the leaves were sprayed with 40% (v/v) C72 CFCM. Sterile water was used as the negative control. One day after application, the eGFP-tagged *B. maydis* spore suspension was inoculated on leaves, the plants were then maintained in greenhouse for 3 days at 25℃ under 14 h photoperiod of fluorescent light and 10 h of darkness. The mycelium of eGFP-tagged *B. maydis* and maize leaves were visualized using a fluorescence microscope (Olympus Corporation, Tokyo, Japan). The experiment was performed in five replicates.

2.7. Antagonistic effect of C72 CFCM on Bipolaris maydis

2.7.1. In vitro tests of C72 CFCM for inhibiting mycelial growth of Bipolaris maydis

The inhibition effect of C72 CFCM against *B. maydis* mycelial growth was determined using a "poisoned" PDA plate test. These media contained different concentrations (0.125, 0.25, 0.5, 1, 1.25, 2.5, 5, 10 and 20% (v/v) dilutions) of C72 CFCM. A 5 mm diameter mycelial plug of *B. maydis* obtained from 5-day-old culture plate was placed on the center of amended PDA plate. No CFCM was added in control plates. Three replicates were set up for each treatment. The diameter of mycelium was measured after incubation at 28℃ for 3–5 days and the inhibition efficacy was calculated. Inhibition rate (%) = (diameter in control - diameter in treatment) / (diameter in control) × 100. The 50% effective concentration (EC50) values for growth inhibition were determined by non-linear regression analysis using GraphPad Prism Software version 8.0.0 (GraphPad, Santiago, California, USA). After growing on the "poisoned" plate for 5 days, the mycelium at the edge of the inhibition zone was observed by a light microscope (ECHO, Santiago, California, USA).

2.7.2. In vitro tests of C72 CFCM for inhibiting spore germination of Bipolaris maydis

The inhibition assay of C72 CFCM on spore germination of *B. maydis* was performed in a 96-well microtiter plate. Briefly, serially diluted CFCM (100 μL) were added to each well at concentrations of 40, 20, 10, 5, 2.5, 1.25, 0.62, and 0.31% (v/v), and the well was filled only with LB broth as controls. Then, 100 μL spore suspension (1×10^5 spores mL^{-1}) was added into each well. The microtiter plates were incubated at 28 °C for 12 h and the percent germination was observed under a light microscope. There were three replicates for each dilution. The counted number of spores was not <200 under three random microscope fields for each replicate. The spore germination standard was defined as the length of the germ tube exceeded the longitudinal length of the spores. The germination rate was calculated as the percentage of the germinated spores to the total spores, and the inhibition rate (%) = (spore germination rate in control - spore germination rate in treatment)/spore germination rate in control × 100. The 50% effective concentration (EC50) values of CFCM against spore germination were calculated by non-linear regression analysis using GraphPad Prism Software version 8.0.0 (GraphPad, Santiago, California, USA). To further investigated the effect of CFCM on *B. maydis* spore germination, the morphological changes of 0.62% and 5% (v/v) CFCM-treated spores were observed by a light microscope.

2.8. Effect of C72 CFCM on the cell membrane permeability of Bipolaris maydis mycelium

The *B. maydis* mycelium taken from a 5-day-old culture plate was immersed in 10% (v/v) CFCM at 25℃ for 1 h. The mycelium treated with liquid LB was used as controls. Then, the mycelium was transferred into 20 μM propidium iodide (PI) in the dark for 10 min. PI can penetrate the compromised membrane of dead cells to stain the DNA. The mycelium was observed under a fluorescence microscope (Olympus Corporation, Tokyo, Japan). The experiment was repeated five times.

2.9. Thermal stability of C72 CFCM

The thermal stability of CFCM was evaluated by investigating the change of the inhibition rate of spore germination. Briefly, CFCM was heat-treated at 45 ℃ for 7 days, 14 days, 21 days and 28 days, and then the spores of *B. maydis* were treated with 1% and 10% heat-treated CFCM at 28 °C for 12 h to calculate the inhibition rate of spore germination. The experiment was repeated three times.

2.10. Production of extracellular lytic enzymes by C72 strain

Enzyme assay were examined by plate assay amended with respective substrate to evaluate the abilities of the C72 strain to produce extracellular lytic enzymes. The activity assay methods of chitinases, β-1,3-glucanases, and cellulases were conducted with reference to the literature (Cherkupally et al., 2017; Madbouly et al., 2020) with slightly changes. The activities were determined by the formation of clear zones around the bacterial colonies, and the experiments were performed with three replicates.

2.11. Gene expression

Quantitative Real-time PCR (qRT-PCR) was used to investigate the expression of maize defense response genes *NPR1* (nonexpressor of pathogenesis-related genes 1), *PR1* (pathogenesis-related protein 1), *PAL* (phenylalanine ammonia-lyase) and *PDF1.2* (plant defensin gene) at 12, 24, 36, and 48 h after spraying 40% CFCM on the leaves of the maize plants under greenhouse conditions mentioned in section 2.4. Sterile water was used as the negative control. Total RNA was extracted from maize leaves using the EasyPure® Plant RNA Kit (Transgen Biotech, Beijing, China). The extract RNA was treated with DNAase and used for cDNA synthesis using a PrimeScript® RT reagent kit (TaKaRa, Dalian, China). The primers used for qRT-PCR were designed using NCBI Primer-BLAST and listed in Supplementary Material Table S2. qRT-PCR was performed using TransStart® Top Green qPCR SuperMix (Transgen Biotech, Beijing, China) on ABI Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The standard amplification protocol was 94℃ for 30 s. Then, the following cycle was repeated 40 times: 95℃ for 5 s, 60℃ for 15 s, and 72℃ for 10 s. All the data were normalized to actin gene expression, the relative mRNA quantities were calculated using the △△Ct method (Schmittgen and Livak, 2008). Each sample had three biological replicates, with three technical replicates for each biological replicate.

2.12. Statistical analyses

The data obtained were subjected to the standard analysis of variance (ANOVA) procedure with SAS software (SAS Institute, Cary, NC, USA). Data was analyzed by ANOVA followed by Tukey's test for post-hoc comparison. All data were checked for normality by the Shapiro-Wilk test before statistical analysis. Differences were considered significant at $P < 0.05$ and respective significant differences marked using different letters. Data are presented as means ± standard error derived from three independent experiments unless otherwise noted.

3. Results

3.1. Screening and identification of bacterial strain C72 against Bipolaris maydis

The antagonistic activity differed significantly among 29 entomopathogenic bacteria (Supplementary Material Table S1). Among these isolates, the inhibition rates of mycelial growth of *B. maydis* were 15.21% − 77.97%, isolate C72 exhibited the highest antifungal activity against *B. maydis*. Physiological and biochemical assays indicated that strain C72 was a Gram-negative bacterium (Supplementary Material Fig. S1). This strain was negative for oxidase and catalase activity, motile, and appeared as non-spore-forming rods (0.6–1.0 μm in width and 2.5–4.0 μm in length). The single colony grew on LB agar plate was circular, convex, regular edges, cream colored and 1.5–3.0 mm in diameter after 3 days at 28℃, the primary phase colonies of C72 were dark blue on NBTA indicator plates (Supplementary Material Fig. S1). The *16S rRNA* and *gyrB* sequences (accession number MT967912 and MW020592) of strain C72 shared over 99% nucleotide sequence identity with *X. budapestensis*. Based on blastn results and phylogenetic analysis (Fig. 1) of these two genes, strain C72 was classified as *X. budapestensis*.

3.2. Determination of broad-spectrum antifungal ability of strain C72

Antifungal activity evaluations of living cells and CFCM of C72 were performed against 17 representative phytopathogenic fungi. By the CFCM-amended plates test, C72 CFCM effectively inhibit the mycelial growth of all tested targets in a dose-dependent way (Table 1 and Supplementary Material Fig. S2). The inhibitory effect of 40% (v/v) CFCM can reach to 100% on all tested fungi strains, while the inhibitory effect of 4% (v/v) CFCM ranged from 58.14% to 90.23% on all tested strains (Table 1). In particular, the CFCM exhibited excellent inhibitory effect against other four different fungal pathogens of maize foliar diseases, only 4% (v/v) CFCM can reduce the mycelial growth by more than 70%, of which *E. turcium* decreased by 86.95%, *R. cerealis* decreased by 83.54%, *B. zeicola* decreased by 82.97% and *C. lunata* decreased by 79.92%. In addition, the bacterial suspension of strain C72 exhibited broad-spectrum antagonistic activity against these fungal pathogens, and the inhibition rates of mycelial growth ranged from 53.08% to 87.01% (Table 1).

3.3. Biocontrol efficacy of C72 strain on SCLB

In the greenhouse trials, both living bacteria and CFCM were tested to evaluate the biocontrol efficacy on SCLB. The results showed that the biocontrol efficacies of 10% and 40% bacterial suspension reached 70.23% and 57.87% respectively after 14 days of inoculation; while the biocontrol efficacies of cell pellets were decreased significantly at the same concentration. Moreover, bacterial suspension and CFCM had significant control efficacies on SCLB, and no significant differences were found between them at the same concentration (Table 2). Therefore, we speculated that the main action modes of strain C72 against *B. maydis* was probably to secrete antimicrobial active substances into the culture supernatant.

In case of CFCM, the control efficacies of 2.5%, 10%, and 40% CFCM were 29.05%, 68.01%, and 77.96%, respectively (Table 2). There was no significant difference in the control efficacy between 10% and 40%, but the efficacies of 10% and 40% were significantly higher than that of 2.5% CFCM. Compared to chemical pesticide treatment groups, the control efficacy of 40% CFCM was comparable to that of 500·mg mL^{-1} chlorothalonil (the control efficacy is 76.49%) but slightly higher than that of 400·mg mL^{-1} carbendazim (the control efficacy is 58.81%). There was no significant difference in control efficacy between 10% CFCM and the two fungicides. Therefore, the 10% and 40% C72 CFCM were subsequently selected to evaluate the control efficacy on SCLB in field trials.

From 2019 to 2020, two independent assays were conducted under field conditions (Table 3). In 2019, the biocontrol efficacy of SCLB by C72 CFCM was assessed and the different concentrations of CFCM were found to have significant control effect on SCLB, the control efficacy of 40% and 10% (v/v) CFCM reached to 67.97% and 57.01% on the 14th day, respectively (Table 3). This efficacy was similar to that of applying 500 mg mL^{-1} chlorothalonil (the control efficacy is 69.51%) and 400 mg mL^{-1} carbendazim (the control efficacy is 57.30%). Besides, the control efficacy of 10% and 40% (v/v) CFCM on the 21th day decreased slightly to 40.08% and 59.15%, respectively (Table 3). But it was still comparable to the fungicide carbendazim (the control efficacy is

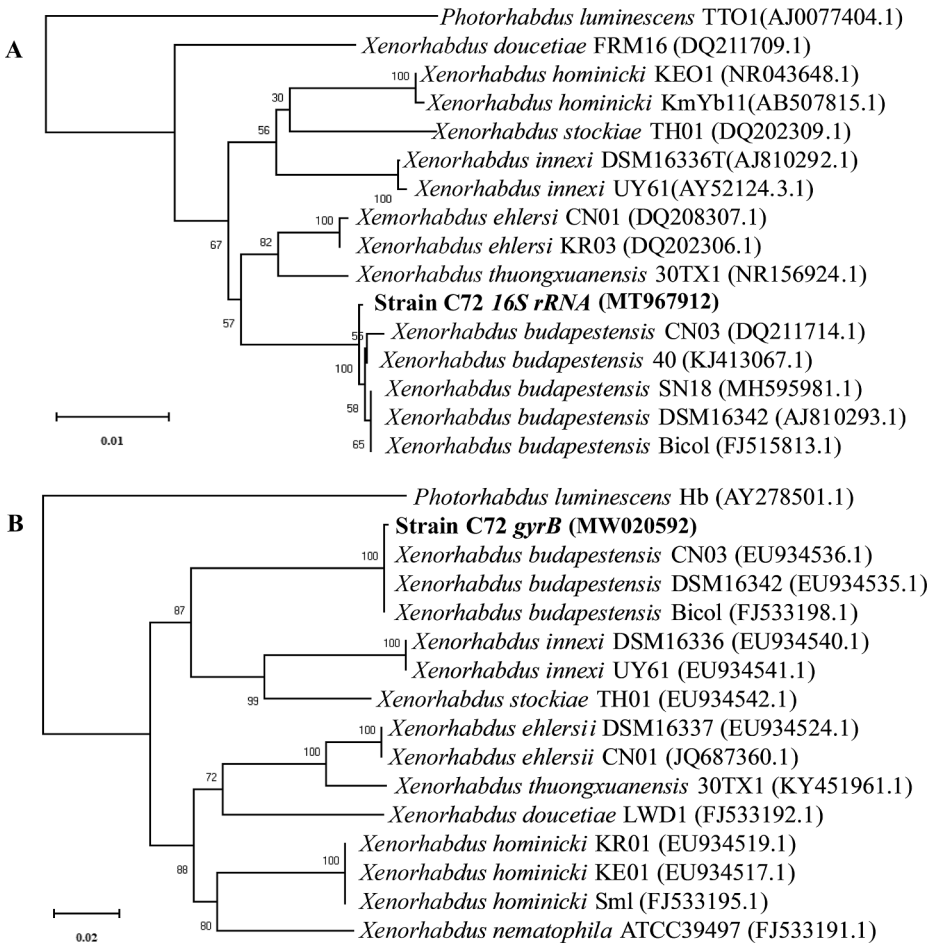

Fig. 1. Neighbour-joining tree based on analysis of partial *16S rDNA (*A) and *gyrB* (B) nucleotide sequences of the strain C72. The evolutionary history was inferred using the Neighbor-Joining method with Maximum Composite Likelihood model in MEGA X. Bootstrap values (%) presented at the branches were calculated from 1000 replications. The scale bar represents the number of substitutions per site.

50.05%). In 2020, 40% and 10% (v/v) CFCM led to average 38.52% and 51.22% disease reduction, respectively. There is no significant difference in disease reduction between 40% (v/v) CFCM and chlorothalonil (the control efficacy is 56.39%), but the control efficacy of 10% (v/v) CFCM was less effective than the fungicide.

3.4. Effect of C72 CFCM on the colonization of Bipolaris. Maydis on maize leaves

In the colonization test, our observations revealed that only a few spores on the surface of the maize leaf treated with CFCM germinated. Even the germinated spores, they obviously failed to penetrate into the cells to achieve colonization. Also, the fully extended hyphae with green fluorescence covered on the non-treated leaves surface and tightly surrounded mesophyll cells, indicating that hyphae entered the cells to successfully colonize (Fig. 2).

3.5. Effect of C72 CFCM on the mycelial growth of Bipolaris maydis

The inhibitory efficacy of C72 CFCM upon *B. maydis* mycelial growth and spore germination showed a dose-dependent response (Table 4). The mycelial growth was nearly completely inhibited on 10% (v/v) CFCM-amended PDA plates, and the 50% effective concentration (EC50) values of the CFCM against the mycelial growth was 0.22% ($R^2 = 0.92$). The growth of mycelium on the low concentration CFCM-amended plate was restricted and disordered, the mycelium taken from the edge of the inhibition zones showed the morphological abnormalities, including irregular swelling and excessive branching (Fig. 3A). Furthermore, the vitality of *B. maydis* mycelium immersed in 10% CFCM for 1 h was examined by fluorescence observation. The CFCM-treated mycelium was stained fluorescent red, which was the result of impaired cell membrane permeability (Fig. 3B), whereas the red fluorescence was barely observable in the mycelium of the control group.

3.6. Effect of C72 CFCM on the spore germination of Bipolaris maydis

After 12 h of incubation at 28℃, the spores of *B. maydis* germinated more than 90% in controls. The inhibition rate reached 98% with 2.5% (v/v) CFCM treatment, and the EC50 values of the CFCM against the spore germination was 0.44% (R2 = 0.99) (Table 4). The spore germination was significantly inhibited in a dose-dependent manner. Meanwhile, the spores treated with 5.0% CFCM for 1 h were re-transferred to the PDA plate, and no spore revived. A series of morphological abnormalities began to appear in the germinated spores treated with 0.62% (v/v) CFCM, including malformation of the germ tube, increased and shortened hyphal branching; while the growth of germ tube was normal in controls, and the morphology of germ tube was smooth and no branching in early germination (Fig. 4A). On the other hand, 5.0% (v/v) CFCM treatment rapidly caused noticeable plasmolysis of conidial cells and shrinkage of the protoplasm within 1 h, and ultimately caused protoplasm degeneration and cytoplasmic constituent leakage due to the damage of plasma membranes (Fig. 4B).

Table 1
Antifungal activity of *Xenorhabdus budapestensis* C72 CFCM and bacterial suspension against different plant fungal pathogens.

Fungal pathogens	Inhibition rate of mycelial growth (%)		
	40% (v/v) CFCM	4% (v/v) CFCM	5 μL bacterial suspension
Valsa ceratosperma	100	90.23 ± 1.92	72.45 ± 2.54
Botryosphaeria dothidea	100	89.64 ± 2.01	68.45 ± 4.37
Bipolaris sorokiniana	100	88.45 ± 0.56	76.84 ± 3.64
Botrytis cinerea	100	87.25 ± 1.23	82.54 ± 1.94
**Exserohilum turcium*	100	86.95 ± 2.67	65.85 ± 2.45
**Rhizoctonia solani*	100	83.54 ± 0.57	73.56 ± 1.57
**Bipolaris zeicola*	100	82.97 ± 0.26	79.01 ± 3.98
Phytophthora capsici	100	82.62 ± 1.67	77.45 ± 2.68
Alternaria solani	100	81.54 ± 1.87	87.01 ± 2.01
**Curvularia lunata*	100	79.92 ± 1.73	57.63 ± 1.84
Magnaporthe grisea	100	77.45 ± 1.03	53.38 ± 2.47
Alternariaalternata	100	69.23 ± 1.98	85.74 ± 3.21
Fusarium oxysporum	100	67.23 ± 1.64	61.84 ± 1.83
Verticillium dahliae	100	64.87 ± 2.13	55.34 ± 0.94
Fusariumgraminearum	100	62.97 ± 2.78	59.46 ± 3.65
Fusarium pseudograminearum	100	60.34 ± 5.32	69.01 ± 5.02
Fusarium culmorum	100	58.14 ± 4.24	62.23 ± 3.76

The asterisk represents the fungal pathogens causing the maize foliar diseases. CFCM: cell-free culture media.

3.7. Thermal stability of C72 CFCM

In this study, the antifungal ability of CFCM after heat treatment was assessed and the results showed that the 1% and 10% (v/v) CFCM treated with high temperature (45 ℃) for 14 days had no difference in inhibiting spore germination compared to untreated CFCM (Fig. 5). The inhibition effect of 1% (v/v) CFCM on spore germination decreased slightly with the treatment time extended to 21 days, whereas no significant difference was found in 10% (v/v) CFCM after thermal treatment for 21 days compared to their 14 days counterparts.

3.8. Extracellular enzyme activities assay

The activity of extracellular enzymes was investigated by examining the production of clear zones in the media supplemented with corresponding substrates. The *X. budapestensis* C72 strain produced chitinase, cellulase and β-1,3-glucanase (Fig. 6). The above results indicated that C72 strain had the potential to degrade the contents of the fungal cell wall, such as chitin, β-1,3-glucan and glucosidic bonds.

3.9. Expression of disease-resistant related genes induced by C72 CFCM

To determine the signaling pathways activated by CFCM at 40% (v/v) concentration, the expression of maize resistance-related genes was analyzed, which involved in the SA or JA/ET pathways (Fig. 7). The expression of *NPR1*, which is a positive regulator of SA-responsive genes, and *PR1*, which is a plant defense related gene, were significantly up-regulated compared to control plants at 12 h − 48 h after CFCM treatment. The expression levels of *NPR1* reached its peak at 12 h with 3.5 times up-regulation and *PR1* reached its peak at 24 h and exhibited 4.1 times up-regulation. The expression of *PAL* gene, which has a crucial role in secondary phenylpropanoid metabolism, was gradually increased from 12 to 48 h after CFCM treatment, and the *PAL* gene was most strongly induced by 3.1 times at 48 h compared to control. For jasmonic acid (JA) / ethylene (ET) signaling, the key gene *PDF1.2* in treated plants was no significant expression difference throughout the experimental period, but was slightly down-regulated at 12 h. Meanwhile, there was no symptom of any necrotic lesions in treated plants, indicating that CFCM treatment did not have an obvious negative effect on maize leaves.

4. Discussion

In recent years, with the adjustment of agricultural industry layout, maize has recently became the largest planted and yield grain crop in China (Wang et al., 2014). Owing to the lack of resistant varieties and implement of agricultural practices such as continuous cropping and straw returning, SCLB is becoming more serious. Meanwhile, the unreasonable use of synthetic chemical pesticides has caused the emergence of fungicide resistant pathogens (Dai et al., 2018). Then, the establishment of complementary tools or alternative measures of the traditional SCLB control strategy and its application in agriculture have gradually become the issues that were worthy of attention in sustainable maize industry. Microbial agents or their metabolites have been universally regarded as environmentally friendly and sustainable biocontrol measures for integrated management of agricultural pests and

Table 2
Control effect of CFCM on southern corn leaf blight in the greenhouse.

		40%	10%	2.5%	Water (Control)	Chlorothalonil(500 mg·L^{-1})	Carbendazim (400 mg·L^{-1})
CFCM	Disease index (DI)	11.46 ± 1.67d	16.66 ± 1.05 cd	37.01 ± 4.33b	52.31 ± 3.52a	12.26 ± 1.56d	21.62 ± 3.76c
	Control effect (%)	77.96 ± 3.88Aa	68.01 ± 3.19Aab	29.05 ± 8.80Ac		76.49 ± 3.16a	58.81 ± 4.85b
Bacterial suspension	Disease index (DI)	15.41 ± 1.95c	22.41 ± 2.71c	40.78 ± 1.22b	52.31 ± 3.52a		
	Control effect (%)	70.23 ± 5.45Aa	57.87 ± 4.67Aa	21.69 ± 5.07Ab			
Cell pellets	Disease index (DI)	31.89 ± 2.15b	47.63 ± 4.85a	53.55 ± 3.24a	52.31 ± 3.52a		
	Control effect (%)	38.80 ± 3.25Ba	9.23 ± 5.91Bb	–			

The disease index (DI) was investigated on the 14th day post inoculation with *B. maydis* spore suspension (1×10^5 spores mL^{-1}). The different lowercase letters in each row indicate significant difference ($P < 0.05$) among various treatments, and the different uppercase letters in each column indicate significant differences ($P < 0.05$) among the three groups by Tukey's multiple range test. CFCM: cell-free culture media.

Table 3
Control effect of CFCM on southern corn leaf blight in the field.

			10% CFCM	40% CFCM	Chlorothalonil (500 mg·L^{-1})	Carbendazim (400 mg·L^{-1})	Water (control)
2019	Disease index (DI)	Day 14	15.58 ± 0.62b	11.66 ± 2.11c	11.04 ± 0.35c	15.46 ± 1.12b	36.27 ± 1.56a
		Day 21	25.69 ± 2.01b	20.19 ± 1.28 cd	19.53 ± 2.15d	24.11 ± 2.32bc	49.58 ± 2.58a
	Control effect (%)	Day 14	57.01 ± 1.70b	67.97 ± 4.54a	69.51 ± 2.03a	57.30 ± 5.33b	–
		Day 21	48.08 ± 3.54b	59.15 ± 4.23a	60.44 ± 5.83a	50.05 ± 5.84b	–
2020	Disease index (DI)	Day 14	31.29 ± 2.76b	24.77 ± 2.83c	22.12 ± 3.24c	–	50.87 ± 0.83a
	Control effect (%)	Day 14	38.52 ± 4.83b	51.22 ± 5.22a	56.39 ± 6.84a	–	–

The disease index (DI) was investigated on the 14th or 21th day post inoculation with *B. maydis* spore suspension (1×10^5 spores mL^{-1}). The different lowercase letters in each row indicate a significant difference ($P < 0.05$) among various treatments by Tukey's multiple range test. CFCM: cell-free culture media.

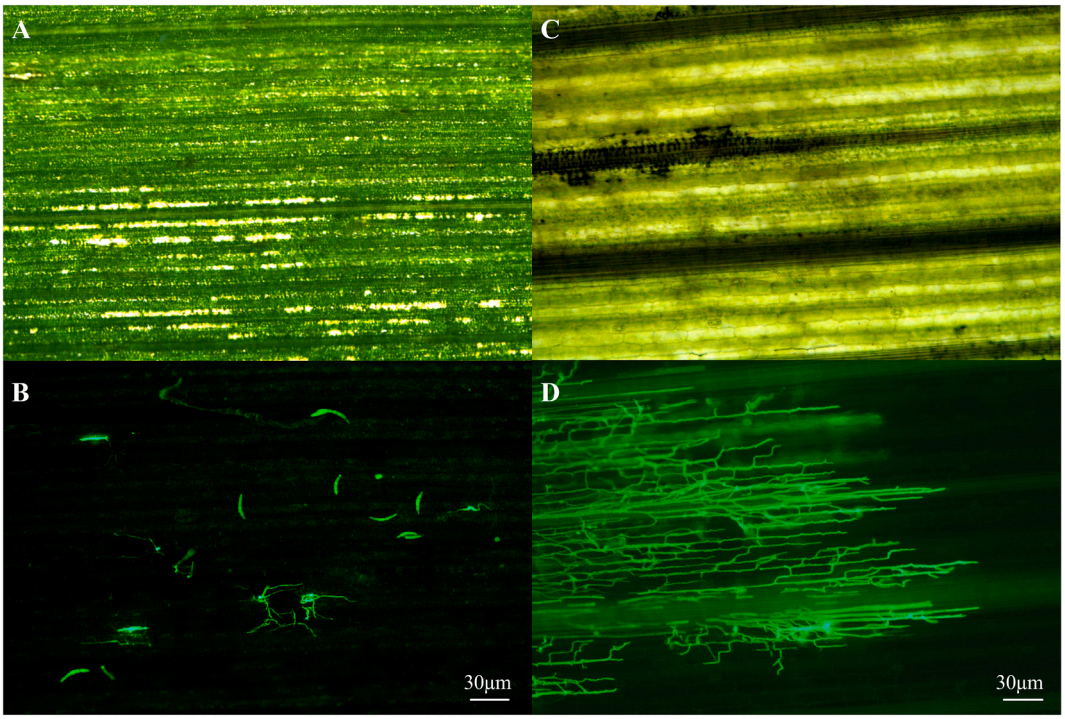

Fig. 2. Effect of *Xenorhabdus budapestensis* C72 CFCM on the colonization of GFP-tagged *Bipolaris maydis* on maize leaves. (A, B) CFCM-treated leaf of maize plants under white light and fluorescence microscope, respectively; (C, D) Not treated leaf of maize plants under white light and fluorescence microscope, respectively.

Table 4
The inhibition effect of various concentrations of *Xenorhabdus budapestensis* C72 CFCM on mycelial growth and spore germination of *Bipolaris maydis*.

CFCM % (v/v)	Inhibition rate of mycelial growth (%)	CFCM % (v/v)	Inhibition rate of spore germination (%)
20	100a	20	100a
10	98.06 ± 1.91a	10	100a
5	94.50 ± 3.46a	5	100a
2.5	87.60 ± 5.33ab	2.5	98.03 ± 1.29a
1.25	78.33 ± 5.56bc	1.25	93.63 ± 2.78a
0.5	65.03 ± 6.71 cd	0.62	68.47 ± 4.62b
0.25	52.73 ± 4.84d	0.31	35.03 ± 10.44c
0.125	21.67 ± 7.78f	0.16	13.67 ± 5.78d

Mean values and standard errors were obtained from three replicates. CFCM: cell-free culture media. The different lowercase letters in each column indicate a significant difference ($P < 0.05$) among various concentrations by Tukey's post-hoc test.

pathogens (Keswani et al., 2019; Köhl et al., 2019). A previous study showed that the bioactive metabolites of *X. budapestensis* DSM 16,342 and *X. szentirmaii* can effectively inhibit the fire blight bacterium *Erwinia amylovora* on apple in phytotron experiments (Vozik et al., 2015). Our results are consistent with the above report, as *X. budapestensis* strain C72 had intensive inhibition effect on mycelial growth of *B. maydis* in dual culture test. Besides, C72 CFCM could effectively protect maize from SCLB under greenhouse and field conditions with biocontrol efficacy was comparable to fungicide treatments (Table 2 and Table 3). The above results demonstrated that entomopathogenic bacteria of the genus *Xenorhabdus* are one of the underexplored sources of bioactive metabolites.

Biological control provides a powerful alternative to the application of pesticides for suppressing plant diseases. However, most of the biocontrol agents have not been widely applied commercially, one of the most important reason is the unstable biocontrol effect in the field (Pliego et al., 2011). The control efficiency of biocontrol agents on plant foliar diseases, especially the use of live microbes as biocontrol agent, is crucially affected by environmental parameters and inoculation conditions like temperature and humidity, inoculation time and growth status of organisms (Le Mire et al., 2016). These factors are closely related to the colonization and reproduction of antagonistic microorganisms and antimicrobial compounds production (Zhang et al., 2017). Similarly, entomopathogenic bacteria are susceptible to external environmental factors. It is difficult for them to survive independently under field conditions, which not conducive to the development of commercial products. In our study, the CFCM of C72 strain exhibited excellent antagonistic activity against *B. maydis* with good thermal stability. When the CFCM was placed at 45 ℃ for at least three weeks to simulate

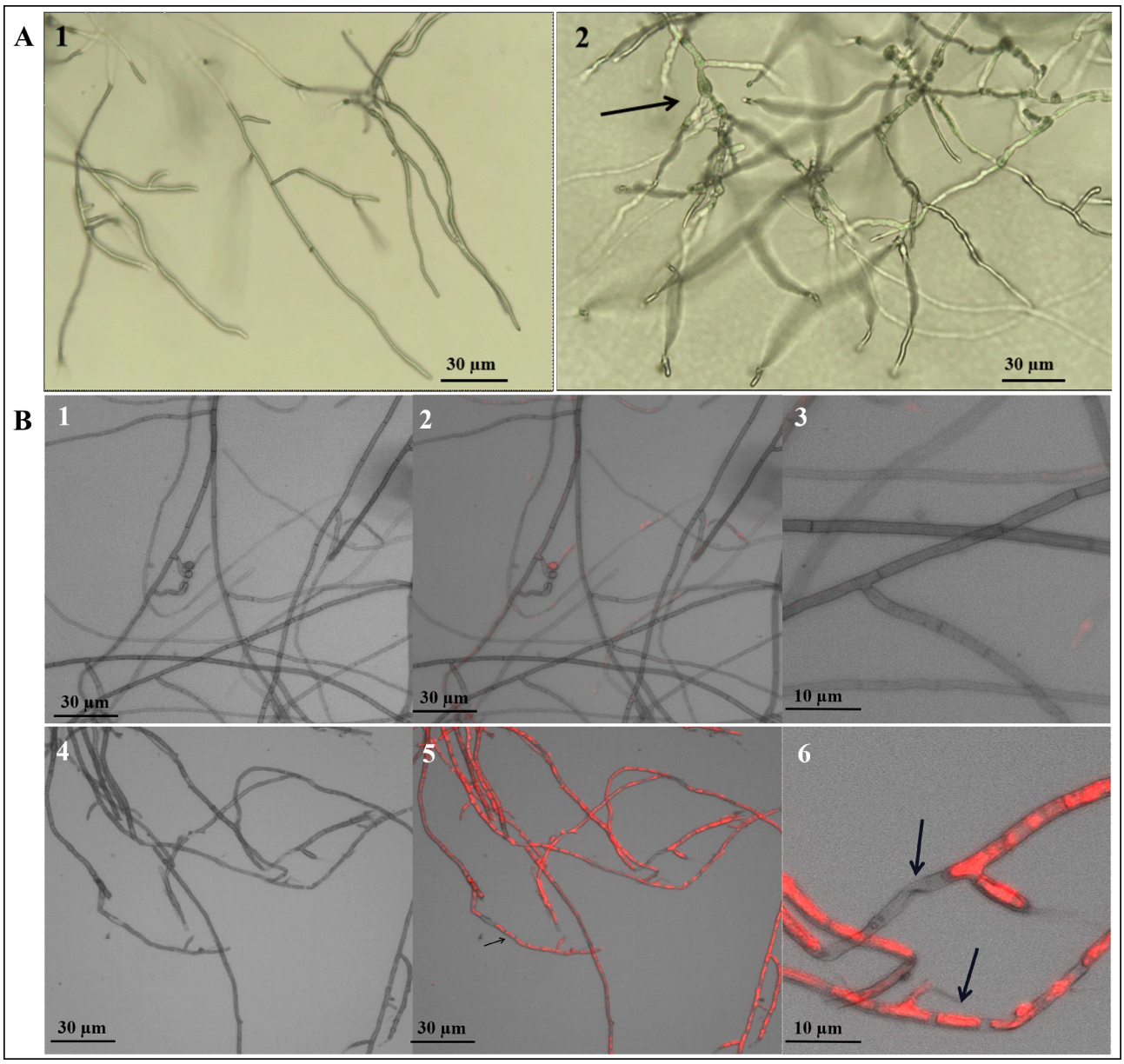

Fig. 3. Effect of *Xenorhabdus budapestensis* C72 CFCM on mycelial growth of *Bipolaris maydis*. (A) The morphological changes of mycelium at the edge of the inhibition zone in "poisoned" PDA plate test. 1: Mycelia of *B. maydis* from control plate, 2: Mycelia of *B. maydis* with irregular swelling and excessive branching (black arrows) from CFCM-amended plate. (B) Propidium iodide (PI) staining to determine the mycelial cell membrane permeability of *B. maydis* by fluorescence observation. 1–3: Mycelia of *B. maydis* form controls; 4–6: Mycelia of *B. maydis* immersed in 10% (v/v) CFCM at 28 ℃ for 8 h. Mycelial cell wall degradation and plasmolysis by CFCM treatment (black arrows). 1,4: Brightfield picture, 2,5: Merge picture, 3,6: Locally magnified view of 2 and 5.

the extreme field environment, the antifungal activity on the spore germination of *B. maydis* was basically unaffected *in vitro* (Fig. 6), indicating that the CFCM had stable properties and could maintain effective antifungal ability in natural environment, which is beneficial to the development of stable preparations for commercial use.

Another feature of potential bacterial agents that eventually enter the market is that they have a broad spectrum of antagonistic activity for controlling a variety of plant diseases, thereby increasing the potential profitability and recognition. In our study, we found that not only C72 CFCM can significantly inhibit mycelial growth of *B. maydis*, but also exhibited a broad antifungal spectrum against various important pathogenic fungi in dual culture assay. Additionally, it has been proved that the cell free supernatant of several *X. budapestensis* strains exerted an exceptionally strong antimicrobial effects on several clinical and zoonotic pathogen isolates (Burgettiné Böszörményi et al., 2015; Xi et al., 2019). Arguably, entomopathogenic bacteria exhibit impressive insecticidal activity after forming a complex symbiosis with nematodes, but the researchers have not yet fully realized the potential of symbiotic bacteria for other applications besides pest management.

One of the first barriers that fungal pathogens have to breach to gain entrance to their hosts and initiate colonization is cell walls (Rodriguez-Moreno et al., 2018). Preventing the successful colonization of *B. maydis* on the leaf surface is the key to protect the maize plants from SCLB. It is a remarkable phenomenon that CFCM treatment restricted the spore germination of *B. maydis* and prevented from fungal hyphae invading parenchyma tissue. This anti-fungal colonization capacity of C72 CFCM is crucial for biocontrol effectiveness, because the inoculated fungal spores were limited to the niche in contact with the blade surface and cannot cause serious SCLB. There are several bodies of evidence which support the fact that some biocontrol agents caused growth inhibition of

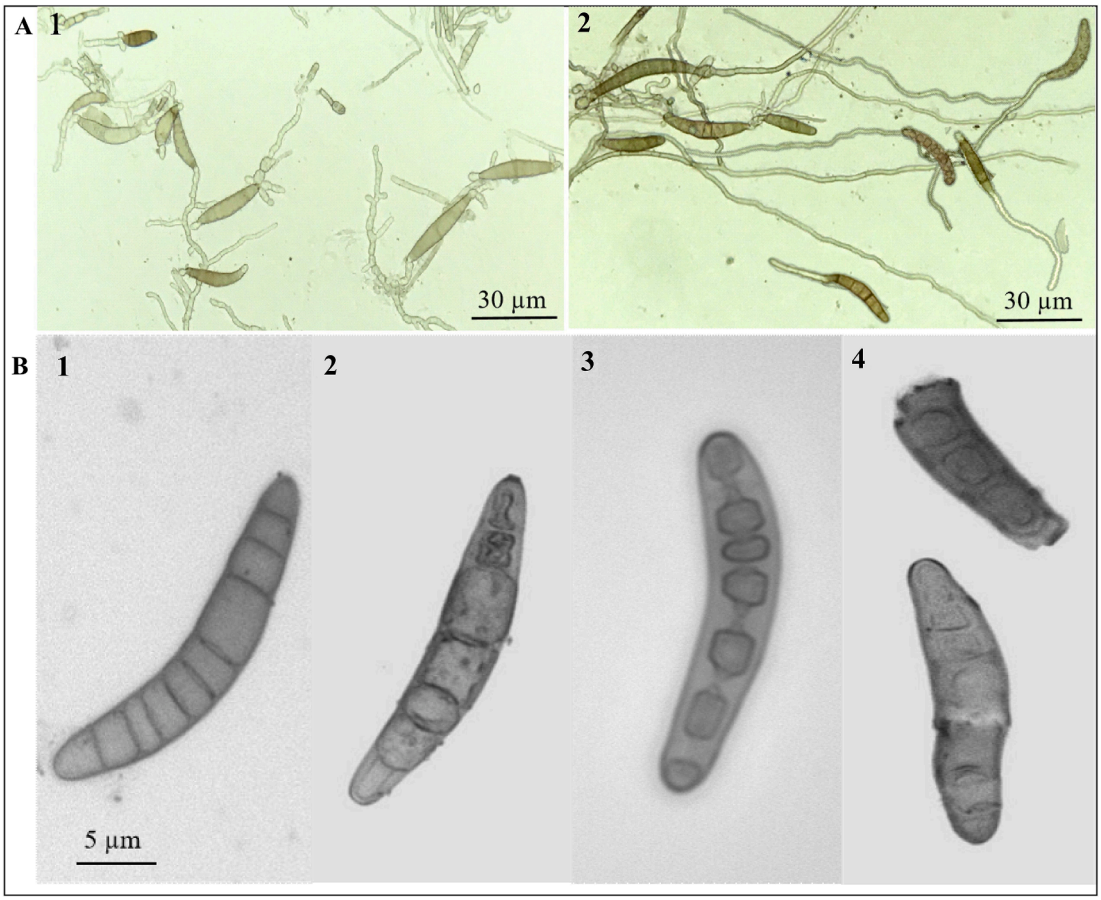

Fig. 4. Effect of 5% (v/v) and 0. 62% (v/v) CFCM of *Xenorhabdus budapestensis* C72 on spore morphology of *Bipolaris maydis*. (A) 1: Abnormally germinated spores by 0. 62% (v/v) CFCM treatment, 2: normally germinated spores in controls without CFCM treatment. (B) The changes of conidial structures of *B. maydis* under 5% (v/v) CFCM treatment, 1–4: 0 h, 0.5 h, 1 h and 8 h after CFCM treatment.

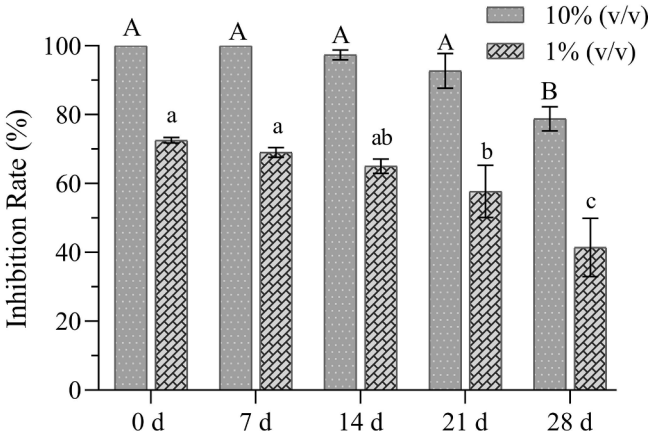

Fig. 5. Thermal stability of C72 CFCM. 1% and 10% (v/v) CFCM was placed in incubator at 45C for 0, 7, 14, 21, 27 days. Thermal stability was assessed by investigating the change of germination rate of *B. maydis* spores was treated with heat-treated CFCM. Each value is the mean of three replicates. Bars represent standard errors of the means. Data in the same concentration indicated by different letters above bars were significantly different ($P < 0.05$) according to Tukey's post-hoc test.

target fungal pathogens and reduced the penetration and colonization on their host plant by secreting a variety of secondary metabolites and/or lytic enzymes (Compant et al., 2013; Haas and Défago, 2005).

Antagonists can display multiple modes of action in plant disease suppression; sometimes different modes act simultaneously, and it is therefore difficult to determine which individual mechanism led to a specific antifungal effect (Di Francesco et al., 2016; Raymaekers et al., 2020). It is generally considered that the basic mode of action for bacteria as biocontrol agents, it could be the production of secondary metabolites with antibiotic properties. Simultaneously, hydrolytic enzymes like cellulase and glucanase, as well as chitinase are likely to expressed in combination to inhibit pathogen growth. In the present study, we found that the antifungal potency of C72 could be partially attributed to production of extracellular enzymes. Microscopic observations demonstrated that the plasmolyzed spores and partially killed hypha of *B. maydis* were appeared after CFCM treatment (Figs. 3 and 4). This might be related to antagonistic compounds produced by *X. budapestensis* impaired cell membrane of spore and mycelium, which eventually caused the extensive intracellular components to leak into the extracellular space through the damaged membrane. Previous studies showed that the extracellaluar proteins extracted form CFCM of *X. bovienii* possessed both exo- and *endo*-chitinase activity and distorted the conidial germination of *B. cinerea* (Chen et al., 1996). Generally, cellulase is similar in structure and enzymatic degradation mechanism to chitinase and can catalyze the degradation of the β-1,4-glycosidic bonds in cellulose (Ferrari et al., 2014). It has been reported that cellulase can be used as an elicitor to trigger plant responses that efficiently protects plants from pathogens (Ma et al., 2015). Besides, the β-1, 3-glucanase is another lysis enzyme that plays an important role in the enzymatic degradation of cell walls of phytopathogenic fungi (O'Brien 2017). In our study, the activity of three above-mentioned fungal cell wall lysis enzymes in *X. budapestensis* C72 strain were verified (Fig. 6).

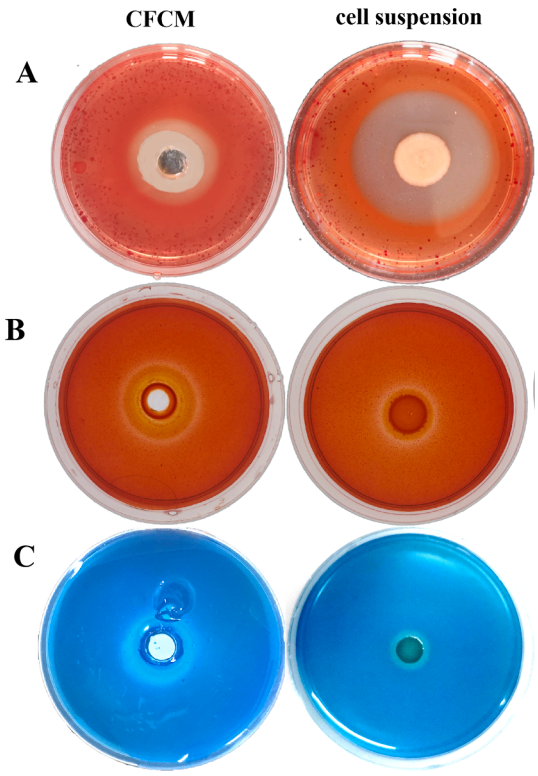

Fig. 6. Enzyme assay of chitinase, cellulase and β-1,3-glucanase produced by *Xenorhabdus budapestensis* C72 Strain. (A) Laminarin plates amended with 0.5% Laminarin (w/v). Clear zones (indication of the β-1,3-glucanase activity) were developed with 0.05% Congo red solution (w/v). (B) Cellulose plates amended with 0.5% carboxymethyl cellulose (w/v) and 0.1% Congo red (w/v). The formation of bright yellow halo indicates the cellulose degradation activity. (C) Chitin plates amended with 0.4% colloidal chitin (w/v) and 0.015% bromocresol purple. The formation of clear zones indicates the chitinase activity. Left side is 100 μL CFCM, right side is 5 μL cell suspension (1×10^7 CFU mL^{-1}) of *X. budapestensis* C72 Strain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

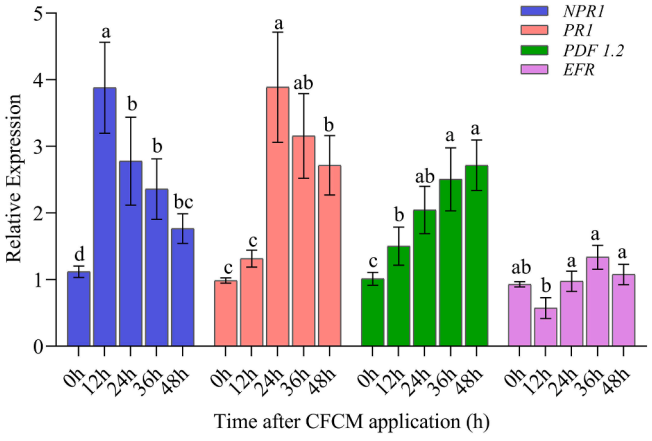

Fig. 7. The expression of resistance-related genes induced by 40% CFCM of *Xenorhabdus budapestensis* C72. Data in the same gene indicated by different letters above bars were significantly different ($P < 0.05$) by Tukey's post-hoc test. *NPR1*: nonexpressor of pathogenesis-related genes 1, *PR1a*: pathogenesis-related protein 1, *PAL*: phenylalanine ammonia-lyase and *PDF1.2*: plant defensin gene.

Therefore, we speculated that these lytic enzymes in C72 CFCM play auxiliary roles in effectively exerting biocontrol effects by decomposing the chitin, and β-glucans.

Stimulating plant defense reactions through elicitors released by biocontrol bacteria such as lysis enzymes, flagellin and antibiotics, which is another common mode of action of biocontrol agents (Ma, 2008; Ma et al., 2015). Hence, we conjectured that strain C72 can also secrete lysis enzymes or other bioactive metabolites as elicitors to trigger systemic disease resistance in an indirect pattern. In our study, the expression of these defense-related genes *PR1* and *NPR1* were concurrently up-regulated in the leaves of CFCM-treated plants (Fig. 7). A larger number of researches have proved that *NPR1* gene is a key node between the SA signaling molecule and *PR1* gene, which plays an active regulatory role in inducing systemic acquired resistance (SAR) in plant host (Kinkema et al., 2000; Wu et al., 2012). The above result and report indicated that the SAR related to salicylic acid (SA)-dependent signaling pathway was activated in maize plant. In addition, *PAL* gene was induced in CFCM-treated maize plant, which plays a crucial role in secondary phenylpropanoid metabolism that produce lignins, coumarins and flavonoids (Chen et al., 2009). A recent study demonstrated that *CaPAL1* acts as a positive regulator of SA-dependent defense signaling to combat microbial pathogens via activating phenylpropanoid biosynthesis pathway in pepper (Kim and Hwang, 2014). Taken together, we put forward the hypothesis that CFCM treatment on maize leaves switched on the SA-dependent SAR and enhanced the biosynthesis of phenylpropanoid-related secondary metabolites to protect maize against *B. maydis*.

In addition to the production of fungal cell wall-hydrolysing enzymes and the stimulation of plant resistance, *Xenorhabdus* spp. are known as a novel source of bioactive secondary metabolites. It has been well documented that those secondary metabolites possess excellent insecticidal and antimicrobial activities against medical infectious vectors, pathogenic bacteria and fungi *in vitro* condition (Shi and Bode 2018). In the case of *X. budapestensis*, the bacteria usually produce multiple antimicrobial compound classes in parallel like PAX lipopeptides (Dreyer et al., 2019), Xenematides (Xi et al., 2019), Fabclavines (Fuchs et al., 2014) and the rhabdopeptide/xenortide peptides (RXPs) (Tobias et al., 2018), and their full antimicrobial potential may be multiplied by synergistic interactions (Böszörményi et al., 2009). Therefore, from the perspective of producing abundant and efficient antimicrobial metabolites, entomopathogenic bacteria like *X. budapestensis* can still be considered as candidate biocontrol agents with high research potential and application value in controlling SCLB and other plant fungal diseases. Even so, the specific types of active natural metabolites produced by C72 and their molecular structure still need further exploration and identification.

In conclusion, CFCM of *X. budapestensis* strain C72 has strong bioactivity against *B. maydis* and multiple other important fungal pathogens *in vitro*, and has excellent protective effect on maize from SCLB by restricting the colonization of *B. maydis*. The full antifungal potential of C72 may be attributed to the production of antifungal antibiotic and cell wall lysing enzymes and activation of plant defenses. In order to achieve subsequent commercial applications, it is necessary to conduct further comprehensive study involving in the identification of microbicidal active substances and the development of practical formulation for the applications of the entomopathogenic bacteria as novel biocontrol agents or biopesticides in agriculture.

CRediT authorship contribution statement

Bo Li: Formal analysis, Writing - original draft. **Lingxiao Kong:** Resources. **Dewen Qiu:** Conceptualization, Supervision. **Frédéric Francis:** Supervision, Writing - review & editing. **Shuangchao Wang:** Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2021.104605.

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