Research

Bacillus subtilis* GB519 Promotes Rice Growth and Reduces the Damages Caused by Rice Blast Fungus *Magnaporthe oryzae

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Abstract

Rice blast disease caused by fungus *Magnaporthe oryzae* (syn. *Pyricularia oryzae*) is one of the most damaging diseases of rice, reducing plant production worldwide. In the present study, *Bacillus subtilis* strain GB519 was identified from the rhizosphere based on predicted signatures of 16S ribosomal DNA and *gyrA* gene and morphological, biochemical, and physiological characteristics. Treated with *B. subtilis* GB519, rice plants exhibited increased germination rate, vigor index, shoot length, shoot fresh weight, and root fresh weight coupled with more production of indole acetic acid, organic phosphorus, and inorganic phosphorus. In culture, GB519 inhibited growth of the following rice fungal pathogens (in order from most effective to least effective): *M. oryzae*, *Ustilagoidea virens*, *Fusarium graminearum*, *F. moniliforme*, *F. oxysporum*, and *Rhizoctonia solani*. Three years of studies showed that, when rice was sprayed with GB519, there were significant reductions in rice blast incidence in both the greenhouse and fields: 70.3 and 62.1% in 2017, 69.9 and 71.6% in 2018, and 75.1 and 75.6% in 2019, respectively. Such reductions were correlated with accumulated hydrolytic enzymes, including amylases, proteases, chitinase, and lipases, and the defense enzyme activity of the total antioxidant capacity, catalase, and superoxide dismutase in rice. Field experiments showed that the biocontrol efficacy of GB519 was similar to that of other biological and chemical fungicides. Our results indicate that *B. subtilis* strain GB519 promoted plant growth and reduced blast disease and suggest that this strain has potential to be used as a biological control agent against rice blast.

Keywords: *Bacillus subtilis* GB519, biocontrol, growth promotion, defense enzymes, *Magnaporthe oryzae*

Rice is one of the most important crops that feeds half of the world population. However, production of rice is limited by harmful fungal microbes such as *Magnaporthe oryzae* (syn. *Pyricularia oryzae*) causing rice blast, *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*) causing sheath blight, *Cochliobolus miyabeanus* (anamorph: *Bipolaris oryzae*)

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causing brown spot, *Ustilaginoidea virens* causing false smut, *Tilletia barclayana* (*Neovossia horrida*) causing kernel smut, and *Cercospora janseana* (teleomorph: *Sphaerulina oryzina*) causing narrow brown leaf spot (Ou 1985). Among them, rice blast is one of the most devastating disease that causes severe crop losses (Dean et al. 2005). Although growing blast-resistant rice cultivars is a cost-efficient strategy, rapid genetic mutations of *M. oryzae* affect the efficiency of newly deployed resistance (Jia et al. 2016; Miah et al. 2012). Fungicides such as isoprothiolane, edifenphos, and iprobenfos have been commonly used to prevent rice blast disease even though fungicide residues can cause environmental pollution and fungicide resistance is well documented in pathogen populations (Du et al. 2011; Lan et al. 2007; Xi et al. 2009; Yuan et al. 2005).

Beneficial microbes such as *Bacillus subtilis* associated with plants can help the plants grow faster and may protect them from the abovementioned harmful microbes (Todorova and Kozuharova 2010). Extensive studies have been done with nitrogen fixation bacteria. The plant provides sugars to the nitrogen-fixing microorganism for nitrogen fixation and the microbe provides fixed nitrogen to the host plant for its growth as an exchange for these carbon sources (Momose et al. 2009). Beneficial microbes such as *B. subtilis* have demonstrated their superiority in their ability to colonize, reproduce, adapt, and help the growth of plants. For example, Harish et al. (2009) demonstrated that *B. subtilis* can control plant pathogens via secreting secondary metabolites. Filippi et al. (2011) discovered that *B. subtilis* promoted plant growth. Furthermore, *B. subtilis* strain T429 (Meng et al. 2015) and *B. methylotrophicus* strain BC79 (Shan et al. 2013) were demonstrated to be useful for reducing damage by *M. oryzae*. However, some mechanisms of antifungal activities and stimulating plant growth are largely unclear.

Understanding the mechanisms underlying *B. subtilis* on plant growth and antifungal activity will benefit the utilization of *B. subtilis* as a potential biological control agent for rice blast disease. The objectives of this study were to (i) specifically identify bacterial strain *B. subtilis* GB519 from the rhizosphere soil, (ii) assess its hydrolytic enzymes and plant growth promotion activities, (iii) evaluate the antifungal activity of GB519 on several other fungi in vitro, (iv) verify its efficacy against *M. oryzae* under greenhouse conditions, and (v) identify the production of defense enzymes.

MATERIALS AND METHODS

Antagonistic bacterial strain and pathogens

Bacterial strain GB519 was screened from healthy rice rhizosphere soils of rice blast disease-affected fields in Gongzhuling, northeastern China. Isolation and screening of antagonistic bacteria was carried out according to Shan et al. (2013). Strain GB519 had been stored at -80°C . Broths were prepared by selecting new single colonies of GB519 from nutrient agar plates, and inoculated in 5 ml of nutrient broth and shaking culture for 48 h at 30°C . Bacterial cell populations were adjusted to 10^8 CFU ml^{-1} with spectrophotometry (Abdallah et al. 2019); then, media consisting of peptone at 10 g/liter, sucrose at 10 g/liter, and agar at 16 g/liter were used in the following tests. Rice blast fungus (*M. oryzae*) was collected and kept at -80°C and sporulated according to the methods of Wang et al. (2015). Other fungi such as *U. virens*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Fusarium graminearum*, *F. moniliforme*, *F. oxysporum*, *Colletotrichum lagenarium*, and *Rhizoctonia solani* used in this study were previously isolated, identified, and kept in our lab.

Identification of bacterial strain GB519

The morphological characteristics as well as biochemical and physiological properties of *B. subtilis* were determined as described by Garrity et al. (2004). The 16S ribosomal DNA (rDNA) and the *gyrA* region were amplified by using two pairs of primers: 27-F (5'-AGAGTTTGTACCTGGCTCAG-3') and 1492-R (5'-GGTTACCTTGTACGACTT-3') (Islam et al. 2016) and *gyrA*-F (5'-CAGTCAGGAAATGCGTACGTCCTT-3') and *gyrA*-R (5'-CAAGGTAATGCTCCAGGCATTGCT-3') (Chun and Bae 2000). PCRs were run with denaturation at 95°C for 3 min; followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; with a final extension at 72°C for 10 min. The gel-purified PCR products were ligated into pESI-T vector (Yeast Biotech, Dalian, China) based on the manufacturer's instructions. The positive recombinant plasmids were purified with SanPrep Column Plasmid Mini-Preps Kit (Sangon Biotech, Dalian, China), digested with the restriction enzyme *Pst*I and separated in a 1× Tris-acetate-EDTA agarose and visualized with Goldview (Solarbio Co., Ltd., Beijing, China). The predicted fragments were gel purified with SanPrep Column DNA Gel Extraction Kit (Sangon Biotech) and were sequenced by Sangon Biotech (Shanghai, China). The sequences were assembled using NCBI (<https://www.ncbi.nlm.nih.gov/>) and a phylogenetic tree of DNA sequence assemblies of 16S rDNA and *gyrA* of GB519 was constructed using the neighbor-joining method in MEGA 7.0 software. The topology of the phylogenetic tree was constructed using 1,000 bootstrap resampling replicates (Kumar et al. 2016).

Hydrolytic enzyme assays and plant growth activities in vivo

The potential production of hydrolytic enzymes of strain GB519 was tested. Amylase activity was identified on 0.2% soluble starch agar plates at 30°C for 48 h (Mishra and Behera 2008). The protease activity was tested on medium containing 1% skim milk at 30°C for 48 h (Yu et al. 2017). Chitinase activity was evaluated using 0.5% chitin-agar medium after the plates were incubated at 30°C for 96 h (Chernin and Chet 2002). Lipase was evaluated using nutrient agar medium supplemented with 1% olive oil emulsion and 0.1% rhodamine B at 30°C for 48 h (Kravchenko et al. 2002). Glucanase activity was tested at 30°C for 72 h, after adding 0.5% β -1,3-glucan in a semisolid nitrogen-free medium (Fan et al. 2002; Renwick et al. 1991). Organic phosphate solubilization was evaluated with 0.02% egg lecithin agar medium for organic phosphorus at 30°C for 72 h. Phosphate solubilization was evaluated with 0.5% $\text{Ca}_3(\text{PO}_4)_2$ agar medium for inorganic phosphorus at 30°C for 3 days (Mehta and Nautiyal 2001). Indole-3-acetic acid (IAA) production was measured by adding Salkowsky's reagent to the supernatant of strain GB519 (Bibi et al. 2012).

The hydrogen cyanide (HCN) was assessed qualitatively on nutrient agar medium supplemented with 0.45% glycine at 30°C for 72 h (Aydi Ben Abdallah et al. 2016; Lorck 1948). Siderophore production was detected on 0.12% chrome azurol-S agar medium at 30°C for 48 h (Shin et al. 2001).

Effect on rice seed

Twenty-five rice seeds were disinfected and treated with broth of GB519 (10^8 CFU ml^{-1}) for 2 h. After seed were dried in air under sterilized conditions, they were placed on two layers of sterilized filter papers with more than 90% humidity in sterilized

petri dishes; appropriate sterilized water was added when necessary. The petri dishes with seed were incubated in an incubator under a day and night cycle of 16 and 8 h, respectively, at 25°C. The two controls were (i) 20 ml of a concentration of 2.5 g/liter (400 times) of liquid of chemical fungicide with 20% iprobenfos-tricyclazole (Beijing Green Agricultural Science and Technology Group Co., Ltd.) and (ii) sterile water. The number of seeds that germinated, root length, and shoot length were measured on the 14th day after treatment. The experiment was conducted with three replicates in each treatment. The vigor index (VI) was calculated using the following formula: $VI = (\text{mean shoot length} + \text{mean root length}) \times \text{germination (\%)} (Abdul-Baki and Anderson 1973)$.

Effect on rice growth

Ten rice seedlings at approximately the fourth leaf stage were planted in plastic pots filled with sterilized paddy soils. Seedlings grown in a greenhouse were sprayed with 20 ml of broth of GB519 at 1×10^8 CFU ml⁻¹ and 25°C. The two controls were (i) 20 ml of a concentration of 2.5 g/liter (400 times) of liquid of chemical fungicide of 20% iprobenfos-tricyclazole (Beijing Green Agricultural Science and Technology Group Co., Ltd.) and (ii) sterile water. Each treatment had three replicates and the experiment was performed three times at different times. The growth characteristics, including the length, fresh weight, and dry weight of shoots and roots, were measured at 30 days after treatment.

In vitro antifungal assays

The confrontation culture method was performed to conform the effects of GB519 on plant pathogen growth and development. GB519 was tested against *M. oryzae*, *U. virens*, *S. sclerotiorum*, *B. cinerea*, *F. graminearum*, *F. moniliforme*, *F. oxysporum*, *C. lagenarium*, and *R. solani*. For evaluation, a 5-mm mycelium plug of each of nine pathogens was placed at the center of a potato dextrose agar plate and drops of 10 µl of broth of GB519 (10^8 CFU ml⁻¹) were placed at two opposite points, each 25 mm from center, and incubated at 25°C in darkness. Control inoculation was a 5-mm-diameter block carrying mycelia of each pathogenic fungus without GB519 in the plate. When mycelial growth in the control plate reached the edge of the plate, the distance between the edge of blast to the edge of bacteria of the treated plate between the two inoculation sites (GB519 and fungal block) was measured. The experiment was performed in three replications and was repeated two times. The formula of inhibition rate was calculated as described by Montealegre et al. (2003).

Use GB519 to control rice leaf blast in the greenhouse

Because GB519 was effective against *M. oryzae* in the lab, 200 rice seedlings at the four-leaf stage were used for efficacy assays using replicated pot experiments (600 by 300 by 30 mm) under greenhouse conditions of 60% humidity and 25°C. Seedlings were first sprayed with 20 ml of broth of GB519 at 1×10^8 CFU ml⁻¹; then, they were treated with a 20-ml conidial suspension of *M. oryzae* at 5×10^5 conidia ml⁻¹ pot⁻¹ using the following scheme: (i) 24 h after GB519 treatment, (ii) 24 h after chemical fungicide with 20% iprobenfos-tricyclazole, and (iii) 24 h after spraying the same volume of water by an artificial sprayer. The experiment was performed with three replications for each treatment and was repeated two times in three different years (2017, 2018, and 2019). The incubation conditions in the greenhouse were maintained at 20 to 30°C with 12 h of light and 12 h of darkness for disease development (Wang et al.

2013). The disease index was measured 7 days after inoculation following the method of Prabavathy et al. (2006). Specifically, the blast scores were recorded according to the 0-to-9 scale rating system described by Wang et al. (2013). Specifically, the index of diseased leaf (IDL) and the biocontrol efficacy were calculated by the following formulas: $IDL (\%) = \frac{\sum(\text{number of different scale} \times \text{value of different scale})}{(\text{all number of test} \times \text{the value of the highest scale})} \times 100$, and $\text{biocontrol efficacy (\%)} = \frac{(\text{IDL of water treatment} - \text{IDL of test treatment})}{\text{IDL of water treatment}} \times 100$.

Use GB519 to control rice blast in the field

To study the biocontrol efficacy of GB519 against rice blast under field conditions in northeastern China, the field experiments were carried out in a rich field with a known history of heavy rice blast infection in Meihekou City, Jilin Province, China (125°77' N, 42°63' E) in 2017, 2018, and 2019. All agronomical practices were carried out normally before rice reached heading stage in the field. The field was divided into 12 plots, and each plot was 100 m². A completely randomized design was arranged with three replicate plots for three treatments. The treatments were as follows: (i) broth of GB519 (1×10^8 CFU ml⁻¹, 15 liters per 667 m²), (ii) 20% iprobenfos-tricyclazole (120 g per 667 m²), and (iii) the control. The disease index and biocontrol efficacy were calculated on the 14th day according to Meng et al. (2015). Specifically, the blast scores were recorded according to the number of diseased panicles, and the rate of diseased panicles (RDP) and the biocontrol efficacy were calculated by the following formulas: $RDP (\%) = \frac{\text{number of diseased panicles}}{\text{total number of panicles}} \times 100$, and $\text{biocontrol efficacy (\%)} = \frac{(\text{RDP of water treatment} - \text{RDP of test treatment})}{\text{RDP of water treatment}} \times 100$.

Rice defense enzyme assays

To elucidate biochemical mechanisms of induced rice defense, we performed the defense enzyme assays of rice seedlings against *M. oryzae* under greenhouse conditions. The greenhouse-grown 30-day-old rice seedlings at the four-leaf stage were used to detect defense enzymes. Each treatment contained three replicates and the experiment was performed three times at different times. The leaves were sampled at different time intervals (0, 6, 12, 24, 36, 48, 72, and 96 h) after inoculation, frozen using liquid nitrogen, and stored in a freezer (−70°C). The experiment was performed in three replications. The activities of defense-related enzymes, including superoxide dismutase (SOD) and catalase (CAT), and total antioxidant capacity (TAOC) were measured with an enzyme assays kit from Jiancheng Bioengineering Institute (category numbers A001-1-2, A007-1-1, and A015-3-1; Nanjing, China).

Statistical analysis

The assay experiments were repeated three times simultaneously. Statistical analysis was performed with the SPSS 19.0 software (SPSS, Chicago, IL, U.S.A.). The value of $P < 0.05$ was used to declare the statistical significance. All data were expressed as mean ± standard deviation.

RESULTS

Identification of the GB519 strain

The bacterial strain GB519 was a Gram-positive bacterium with a peritrichous, single rod cell. On nutrient agar medium,

the GB519 formed creamy white colonies, with a matte surface and rough colony margins (Supplementary Fig. S1A). Scanning and transmission electron microscopy showed that GB519 cells are rod shaped (Supplementary Fig. S1B and C). Also, the phylogenetic trees derived from the 16S rDNA (MT271920.1) and *gyrA* (MT294310.1) sequences of strain GB519 were clustered with other *B. subtilis* strains (Supplementary Figs. S1D and S2A and B). Furthermore, the physicochemical indexes of strain GB519 were tested, with results in Supplementary Table S1. Taken together, these data indicate that GB519 belongs to *Bacillus subtilis*.

The extracellular enzyme activity and plant growth-promoting ability

The *B. subtilis* GB519 strain was verified to produce amylase, protease, chitinase, and lipase on 0.2% soluble starch, skim milk, chitin, and polyvinyl alcohol plus olive oil agar medium, respectively (Fig. 1A to D). The *B. subtilis* GB519 strain showed clear zones around colonies on organic phosphorus and inorganic phosphorus agar medium (Fig. 1E and F). Furthermore, the IAA plant growth hormone was detected 48 h after incubation (Fig. 1G). All of these observed characteristics suggest that GB519 promotes plant growth even though glucanase activity, HCN, and siderophore production were not detected.

The effect on rice seed germination and seedling vigor

The germination rate of rice seed treated with *B. subtilis* GB519 was 90.2%, which was 4.6% higher than water control

and 62.5% higher than that in the iprobenfos-tricyclazole treatment (Table 1). Similarly, the highest VI was 6,719.2 for rice seed treated with GB519 (Table 1), and it was significantly higher than the water control and iprobenfos-tricyclazole treatment, increased by 33.1 and 913.2%, respectively. These results suggest that GB519 improves germination and vigor of rice seedlings.

The effect on rice growth under greenhouse conditions

Rice treated by *B. subtilis* GB519 had higher growth compared with treatments using iprobenfos-tricyclazole or the water control. Treatment with GB519 produced shoot length, shoot fresh weight, and root fresh weight of 186.3 mm plant⁻¹, 115.9 mg plant⁻¹, and 47.0 mg plant⁻¹, corresponding to an increase of 10.6, 46.5, and 76.7% above water control, respectively (Table 2).

Antifungal activity

Nine different plant fungal pathogens were used to verify the antifungal activity of *B. subtilis* GB519. Inhibition diameters and ratio are shown in Figure 2 and Supplementary Table S2. The GB519 inhibited the mycelial growth of all nine fungal pathogens, including the rice-disease-causing agents *M. oryzae*, *U. virens* (87.4%), *F. graminearum* (67.8%), *F. moniliforme* (60.2%), *F. oxysporum* (55.0%), and *R. solani* (55.0%), at varying degrees within 40.0 mm with inhibition ratios greater than 55.0%. Among them, the strongest inhibitory effect of GB519 was against *M. oryzae*, with an inhibition ratio of 89.7%.

FIGURE 1

Photographic presentations of tested enzymes, phosphate solubilization, and indole-3-acetic acid (IAA) produced by strain GB519. **A**, Amylase activity is shown as a clear zone formation on 0.2% soluble starch agar plates. **B**, Protease is shown as a clear zone formation around the colony on medium containing 1% skim milk. **C**, Chitinase was evaluated and the zones of chitin clearing occurred around the colonies after incubated at 30°C for 96 h on 0.5% chitin-agar medium. **D**, Lipase was evaluated and presented as a clear pink halo exhibited on the plate after incubation at 30°C for 48 h on nutrient agar medium supplemented with 1% olive oil emulsion and 0.1% rhodamine B. **E**, Organic phosphate solubilization was evaluated with a translucent milky white growth using 0.02% egg lecithin agar medium for organic phosphorus. **F**, Phosphate solubilization was evaluated with clearing zones around the colony using 0.5% Ca₃(PO₄)₂ agar medium for inorganic phosphorus at 30°C for 3 days. **G**, IAA measured as a positive reaction (P) had pink color compared with the negative control (N) in broth of GB519 after Salkowsky's reagent was added to the supernatant of strain GB519.

Biocontrol of rice leaf blast by GB519 in greenhouse experiments

Because GB519 was the most inhibitory to *M. oryzae*, we examined the use of GB519 to control rice leaf blast by measuring

the disease index after GB519 treatments under greenhouse experiments in 3 years. The disease index of rice blast disease treated with GB519 was 22.9 in 2017, 25.0 in 2018, and 16.9 in 2019, which was significantly lower than that of the water control (77.4 in 2017, 83.0 in 2018, and 67.9 in 2019), respectively

TABLE 1

Effects of GB519 on rice seed germination and seedling vigor^x

Treatment ^y	GR (%)	Increase of GR (%)	VI ^z	Increase of VI (%)
Water control	86.4 ± 4.2 a	–	5,100.1 ± 545.3 b	–
GB519	90.2 ± 2.0 a	4.6	6,719.2 ± 654.1 a	33.1
Iprobenfos-tricyclazole	55.5 ± 7.9 b	–35.4	663.2 ± 230.1 c	–86.6

^x GR = germination rate and VI = vigor index. Means ± standard error followed by the same letter do not differ significantly according to Duncan's multiple range test at $P < 0.05$.

^y Rice seed with different treatments were placed on two layers of sterilized filter papers and incubated in an incubator under a cycle of 16 h (day) and 8 h (night) at 25°C; then, the data of seed germination, shoot length, and root length were collected on the 14th day.

^z VI was calculated using shoot length, root length, and GR.

TABLE 2

Growth promotion activity of GB519 on rice under greenhouse conditions^z

Treatment	Shoot length (mm)	Shoot FW (mg/plant)	Shoot DW (mg/plant)	Root length (mm)	Root FW (mg/plant)	Root DW (mg/plant)
Water control	168.5 ± 1.9 b	79.2 ± 20.7 b	24.8 ± 5.5 a	78.4 ± 19.9 a	26.6 ± 7.8 b	11.4 ± 0.4 a
GB519	186.3 ± 8.9 a	115.9 ± 10.0 a	27.4 ± 1.8 a	99.3 ± 5.0 a	47.0 ± 9.9 a	12.4 ± 1.6 a
Iprobenfos-tricyclazole	159.5 ± 8.2 b	75.3 ± 0.7 b	23.9 ± 1.9 a	76.1 ± 21.6 a	29.3 ± 1.9 b	13.5 ± 1.3 a

^z Growth characteristics, including the length, fresh weight (FW), and dry weight (DW) of shoots and roots, were measured on 30th day, after rice seedlings were treated at approximately the fourth-leaf stage in a greenhouse. Means ± standard error followed by the same letter do not differ significantly according to Duncan's multiple range test at $P < 0.05$.

FIGURE 2

In vitro inhibition by strain GB519 against nine plant pathogens: *Magnaporthe oryzae*, *Ustilagoideia virens*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Fusarium graminearum*, *F. moniliforme*, *F. oxysporum*, *Colletotrichum lagenarium*, and *Rhizoctonia solani*. For evaluation, a mycelium plug of a pathogenic fungus was placed at the center of a potato dextrose agar plate and drops of 10 µl of broth of GB519 (10^8 CFU ml⁻¹) were placed at two opposite points with the plug as center, then incubated at 25°C in darkness. The distance from the two inoculation points of GB519 to fungal plug was measured after mycelial growth in the control reached the edge of plate.

(Table 3). There was no significant difference in disease index between GB519 and iprobenfos-tricyclazole treatments. However, the GB519 treatment had a higher biocontrol efficacy of 70.3, 69.9, and 75.1%, respectively, in the 3 years, which was similar to that of the chemical fungicide (Table 3).

Biocontrol of rice blast disease by GB519 in field experiments

In field experiments, the application of *B. subtilis* GB519 reduced the spread of rice blast disease in 2017, 2018, and 2019. The disease index of rice blast with GB519 was 1.95 in 2017, 2.47 in 2018, and 2.10 in 2019, and the biocontrol efficacy was 62.1, 71.6, and 75.6%, respectively. The treatments with GB519 were as effective as the 20% iprobenfos-tricyclazole at 120 g per 667 m² during the 3 years of experiments (Table 3).

Induction of defense enzymes in rice after blast infection

The TAOC and enzymatic activity of the CAT and SOD increased 6 h after inoculation and reached the maximum at 36 h for TAOC, 24 h for CAT, and 24 h for SOD after rice plants were sprayed with *B. subtilis* GB519 (Fig. 3). There were significantly higher activities in the GB519 treatment than iprobenfos-tricyclazole and water control treatments at 12 and 24 h after inoculation. In rice plants treated with GB519 and *M. oryzae*, TAOC increased 1.5×, CAT increased 3.5×, and SOD increased 1.8× compared with the control, suggesting that transient inductions of these defense enzymes are responsible for reducing blast disease damages.

DISCUSSION

Biological control has become an efficient, alternative, and ecologically friendly method for plant disease management (Kumar et al. 2017; Niu et al. 2013). We identified and determined that the *B. subtilis* GB519 strain could be used as an effective biological control agent to prevent rice blast disease. We showed that *B. subtilis* GB519 accelerated plant growth in vitro by producing more IAA, inorganic phosphorus solubilization, and extracellular lipase (Table 2; Fig. 2). Our findings are consistent with the notion that *Bacillus* spp. may play important roles in supplying more soluble phosphorus to plants (Beneduzi et al. 2008; Goteti et al. 2013; Jorquera et al. 2008). Additionally, we also demonstrated that GB519 solubilized

phosphate and promoted plant growth similar to that of a plant growth stimulator. Furthermore, the increased accumulations of IAA could accelerate nutrient movements that can benefit phosphate utilization for plant growth. Previously, *Bacillus* spp. such as *B. amyloliquefaciens* PPB4 (Islam et al. 2016), *B. cereus* S42 (Aydi Ben Abdallah et al. 2016), and *B. methylotrophicus* H8 (Almoneafy et al. 2012) were also reported to produce IAA at concentrations of 50.18, 26.49, and 59.56 µg ml⁻¹, respectively, which is similar to what we found with *B. subtilis* GB519.

A number of *Bacillus* strains, including GB519, were capable of promoting plant growth and also producing biofilms on plant surfaces, enabling antagonistic activities against various plant pathogens (Chen et al. 2013; Gao et al. 2015; Lin et al. 2017). For example, *B. methylotrophicus* BC79 (Shan et al. 2013) and *B. cereus* (Tokpah et al. 2016) were shown to reduce blast disease, and *B. pumilus* GLB197 reduced grapevine downy mildew (Zhang et al. 2017). In this study, we also found abilities of *Bacillus* strains pertaining to biofilms (data not shown). Together, these findings support our hypothesis that GB519 is a good candidate to control rice blast fungus.

Plants utilize multiple defense pathways, including reactive oxygen species and antioxidant defense pathways, to fight off stresses (Hasanuzzaman et al. 2020). Previously, defense enzymes, including protease, chitinase, and lipase, were shown to degrade and lyse pathogenic fungal cells (Bibi et al. 2012; Muleta et al. 2007; Thampi and Bhai 2017; Tiru et al. 2013). Numerous *Bacillus* spp. were effective to control plant diseases on various crops, and mechanisms were largely unclear (Chithrashree et al. 2011). In the present study, GB519 was shown to accelerate the production of plant defense enzymes CAT, SOD, and TAOC and to inhibit *M. oryzae* growth and infection. The enzymes such as CAT, SOD, and TAOC are known to be mainly involved in the antioxidant defense pathway. These findings suggest that GB519 activates the antioxidant defense pathway in order to restrict *M. oryzae* growth and infection.

In the present study, we demonstrated that the severity of rice blast disease was reduced by using GB519 during 3 years under greenhouse and field conditions (Table 3). The suppression of rice blast by spraying GB519 was as effective as that by spraying the chemical fungicide iprobenfos-tricyclazole. Our results are consistent with previous studies. *B. subtilis* T429 dry flowable had no significant differences in disease biocontrol efficacy compared with 75% tricyclazole (Meng et al. 2015). Filippi et al. (2011) showed that spraying Rizo-52 2 days before pathogen inoculation significantly suppressed leaf blast by 86%. Rice plants treated with

TABLE 3

Biocontrol efficacy of GB519 against rice blast disease under greenhouse and field conditions^z

Treatment	2017		2018		2019	
	Disease index	Biocontrol efficacy (%)	Disease index	Biocontrol efficacy (%)	Disease index	Biocontrol efficacy (%)
Greenhouse						
Water control	77.4 ± 3.1 a	—	83.0 ± 1.7 a	—	67.9 ± 2.8 a	—
GB519	22.9 ± 2.9 b	70.3	25.0 ± 2.3 b	69.9	16.9 ± 2.2 b	75.1
Iprobenfos-tricyclazole	24.5 ± 3.4 b	68.3	21.4 ± 2.1 b	74.2	18.0 ± 1.4 b	73.5
Field						
Water control	5.16 ± 0.62 a	—	8.79 ± 0.73 a	—	8.59 ± 0.58 a	—
GB519	1.95 ± 0.18 b	62.1	2.47 ± 0.30 b	71.6	2.10 ± 0.24 b	75.6
Iprobenfos-tricyclazole	1.71 ± 0.07 b	66.5	2.33 ± 0.40 b	73.3	2.19 ± 0.25 b	74.3

^z Seedlings with approximately four leaves in a greenhouse and plants in a field were used for efficacy assays: (i) 24 h after GB519 treatment, (ii) 24 h after chemical fungicide with 20% iprobenfos-tricyclazole, and (iii) 24 h after spraying the same volume of water by an artificial sprayer. Disease indices of seedlings and plants were assessed 7 and 14 days after inoculation, respectively. Means ± standard error followed by the same letter do not differ significantly according to Duncan's multiple range test at *P* < 0.05.

Rizo-46 and Rizo-55 greatly increased enzymes activities of peroxidase, β -1,3-glucanase, and chitinase that were correlated with suppression of leaf blast disease. Furthermore, a bit of difference in GB519 biocontrol efficacy compared with the chemical iprobenfos-tricyclazole among the 3 years might have resulted from the

effect of climate conditions such as the temperature, sunshine, and moisture, which should be considered as important affecting factors when using biocontrol products.

In the present study, we showed significant increases in the defense enzymes—1.5 $\times$ for TAOC at 36 h, 3.5 $\times$ for CAT at

FIGURE 3 Enzymatic activity of rice plants with different treatments on **A**, total antioxidant capacity (TAOC); **B**, catalase (CAT); and **C**, superoxide dismutase (SOD). Lowercase letters: a = *Magnaporthe oryzae* was sprayed 24 h after GB519 treatment; b = *M. oryzae* was sprayed 24 h after chemical fungicide with 20% iprobenfos-tricyclazole; and c = *M. oryzae* was sprayed 24 h after the same volume of water. Error bars show standard deviations for triplicate assays. The x-axis indicates different time points after inoculation and y-axis indicates enzymatic activity level. Values in the same column followed by the same letter are not statistically different by Duncan's multiple range test ($P < 0.05$).

24 h, and 1.8× for SOD at 24 h—compared with that of the control in rice plants first treated with GB519 and then inoculated with *M. oryzae*. The results demonstrated that these increased enzymatic activities affected plant growth and suggested that they were correlated with rice blast suppression under greenhouse conditions.

In conclusion, we showed that GB519 can promote plant growth and prevent rice blast disease as effectively as one of the most-used fungicides and also demonstrated that GB519 can inhibit nine plant pathogens, including *R. solani* (teleomorph: *Thanatephorus cucumeris*) causing sheath blight, *F. graminearum* and *F. oxysporum* causing rice blight, *F. moniliforme* causing rice bakanae, and *U. virens* causing false smut, in addition to *M. oryzae*. This suggests that GB519 is a broad-spectrum biocontrol agent for rice diseases. We also presented evidence of inhibitory effects on pathogens *C. lagenarium*, *Botrytis cinerea*, and *S. sclerotiorum* causing vegetable diseases, which suggests that GB519 has potential to be a broad-spectrum biocontrol agent. We showed that GB519 hijacked the antioxidant defense pathway to prevent the infections by *M. oryzae*. Further studies are required on topics such as methods for large-scale production, formulation, preservation conditions, shelf life and application, the mode of action, phytotoxicity, safety, and cost efficiency under both greenhouse and field conditions.

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