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Biocontrol potential of two deep-sea microorganisms against gray blight disease of tea

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Abstract

Background Gray blight is among the most destructive diseases that affect tea plants worldwide. In this study, microorganisms from deep-sea sediment samples for those with antagonistic effects were screened against gray blight caused by *Pestalotiopsis theae*.

Results Thirty-two and twenty-eight morphologically different deep-sea bacteria and fungi were isolated, respectively. Isolates B5 and A65 clearly inhibited the pathogens in vitro and were prepared as wettable agent powders for evaluation in micro-plot field trials. Foliar application of the 48-h culture of B5 (1×10^8, 2×10^7, 1×10^7 colony-forming units (CFU)/ml) significantly reduced the incidence of gray blight disease. Compared to the untreated control, spraying with B5 inhibited gray blight disease by 78.57%. Isolate B5 was identified as *Bacillus subtilis* B5 in morphological and 16S rDNA sequence analyses. The foliar application of 7-day cultures of A65 (1×10^8, 2×10^7, 1×10^7 CFU/ml) significantly reduced the incidence of gray blight disease. A65 (10^8 CFU/ml) inhibited gray blight disease by 75.46% and was identified as *Paecilomyces lilacinus* A65 in morphologically and internally transcribed spacer sequence analyses.

Conclusions These candidate microbial pesticides may inhibit gray wilt in tea, replace chemical pesticides' use without causing environmental pollution, and promote the development of green agriculture.

Keywords Gray blight, *Paecilomyces lilacinus*, *Bacillus subtilis*, Biocontrol agent, Wettable powder, Field trial

Background

Tea is an important economic crop (Arafat et al. 2017) and an intensively managed perennial monoculture crop cultivated on large- and small-scale plantations (Roy et al. 2016). The gray blight of tea, caused by *Pestalotiopsis theae*, is among the most destructive foliar diseases that affect tea cultivation. Among leaf blights, gray blight leads to economic losses and has been reported in all major tea-growing countries (Wang et al. 2019a, b). This disease generally affects the mature foliage, bare stalks, and young shoots of tea plants (Tsai et al. 2021) and is typically aggravated under high-temperature and high-humidity conditions, thereby resulting in poor plant growth and decreased tea yields (Kumar et al. 2016).

Various fungicides, with different modes of action, are used to control leaf diseases in tea (Deliere et al. 2010). Although fungicides have shown promising results in controlling foliar pathogens, they have serious limitations, such as drug resistance in pathogens and pesticide residues (Sowndhararajan et al. 2013). Thus, more environmentally friendly methods are needed to control diseases and enable sustainable agriculture (Acero et al. 2011). The demand in the tea industry to either exclusively utilize biological products in disease management or reduce chemical usage through supplementation with

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biological products in integrated management practices is increasing (Vijayan et al. 2016). Biological control of foliar fungal disease in tea using antagonistic microorganisms is not widely performed but has shown potential in a previous study (Idris et al. 2020).

Compared to terrestrial microbial resources, marine microbial resources are abundant and diverse. The ecological, physiological, and genetic characteristics, as well as the development and utilization of marine microorganisms, have been increasingly examined (Ameen et al. 2021). A previous study demonstrated that marine organisms under specific environmental conditions, particularly microorganisms, exhibit unique structures and wide species diversity (Micha-Screttas 2008). Hence, active substances produced by marine microorganisms may exhibit greater biological resistance than that produced by microorganisms on land. Marine organisms show broad application prospects for developing biocontrol agents (Zuo and Kwok 2021). Therefore, alternatives to chemical pesticides may be developed from marine microorganisms.

In this study, marine microorganism strains with potential, as microbial pesticides were identified and proposed the microbial formulations of B5 and A65. The effects of the microbial agents were verified in field experiments.

Methods

Isolating and screening the biocontrol fungi/bacteria for antagonistic activity gray blight disease

The test strains were derived from deep-sea sediment samples collected during the 21st scientific expedition of Dayang Yihao, and stored at − 80 °C for further study. The 16S/ITS rRNA sequencing were carried out with the help of the Bioray Biotechnology Co., Ltd, Xiamen, China. A sequence similarity search was performed using GenBank BLASTN. (Combest et al. 2021).

All the isolated test strains were subjected to primary screening against the pathogens; *P. theae*, *Colletotrichum gloeosporioides*, *Phoma* sp., *Neofusicoccum* sp., *Alternaria* sp. and *Pestalotiopsis* sp. on PDA medium. In the control group, in absence of inoculation biocontrol fungi/bacteria, only indicator fungi were inoculated on the plate, and their records were observed daily. The inhibition rate is calculated according to Formula (1). All the phytopathogens of tea were isolated from the naturally infected tea leaves in Xiamen.

$$\text{Inhibition } \% = (r_0 - r)/r_0 \times 100, \quad (1)$$

where r_0 is the radial growth of pathogen (indicator fungi) without antagonist, and r represents the radial growth of pathogen (indicator fungi) with antagonist. Positive control: Chemical fungicides (chlorothalonil), untreated control: Sterile double distilled water.

Preparation of biocontrol agent for A65 and B5

A65 was inoculated into potato dextrose broth liquid medium according to 2% inoculum and cultured for 7 days at 28 °C, 180 rpm. After fermentation, the liquid was filtered, using double gauze, to remove the mycelium, and then the filtrate was centrifuged at 10,000 rpm and 4 °C for 5 min to obtain A65 spore precipitates, and the freeze-dried spore precipitate was crushed into powder. B5 was inoculated into Luria–Bertani (LB) culture according to the inoculum of 2% and cultured for 2 days at 37 °C, 180 rpm. A total of 5 g of diatomite was added to the 450 ml bacterial solution, then shake and mixed at 37 °C, 180 rpm for 15 min. Then, the above mixture was centrifuged at 4 °C, 10,000 rpm for 5 min, and the mixed sample was collected as precipitate. Finally, the freeze-dried precipitate was crushed into powder.

Subsequently, the appropriate wetting agents, dispersants, protective agents, and other wettable powder compositions were select and adjusted the appropriate proportion of different components in it.

Field experiment

All the field studies were carried out in the tea plantations of Lianhua Village, Tong'an District, Xiamen, Fujian Province, from July to September 2020. The microplot experiment was conducted, using a complete randomized block design. The entire field experiment site was divided into 24 experimental plots, with a one-meter isolation area between each plot. The 24 experimental plots were divided into 8 groups, including a positive control group, a negative control group, an experiment group, and a blank control group, each group was investigated three times. Specific treatment methods for each group are shown in (Table 1).

Before the B5/A65 bacterial agent was applied to the field experiment, the number of viable bacteria in the

Table 1 Treatment of different groups

Group	Treatment method
1	Biocontrol agent (A65, 1×10^8 CFU/ml)
2	Biocontrol agent (A65, 2×10^7 CFU/ml)
3	Biocontrol agent (A65, 1×10^7 CFU/ml)
4	Biocontrol agent (B5, 1×10^8 CFU/ml)
5	Biocontrol agent (B5, 2×10^7 CFU/ml)
6	Biocontrol agent (B5, 1×10^7 CFU/ml)
7	Chemical fungicides (chlorothalonil)
8	Untreated control (Sterile double distilled water)

microbial agent was calculated by dilution spread plate technique to verify the survival of the microbial agent. Then, the B5/A65 bacterial agent was diluted with water to the working concentration. For the positive control group, 1500 g of 75% chlorothalonil wettable powder (Fujian Kefeng Pesticide Co., Ltd, 1125 g of active ingredients) per hectare, was diluted with 750 l of water. Approximately 200 ml of B5/A65 biocontrol agent was sprayed in each plot. The above operation was repeated twice, and the interval between the two applications was 10 days. The incidence was recorded, and the disease index and control effect were evaluated according to the following formula and the disease index table. The relative control effect and the disease index were determined by the two equations:

$$\text{Disease index} = \left[\sum \left(n_j \times l_i\right)\right] \div (n \times l_m) \times 100 \quad (2)$$

$$\text{Relative control effect (\%)} = (D_0 - D) \div D_0 \times 100 \quad (3)$$

where l_i represents the disease index level, n_i represents the number of diseased leaves at the corresponding level; l_m was the highest level value, n was total number of leaves, D_0 was disease index in control, D was disease index of each treatment.

Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) (SPSS 9.0; SPSS Inc., Chicago, IL). The significance of effects of treatments was determined by the magnitude of F value ($P < 0.05$), and the treatment means were separated by Duncan's multiple range test. Values are expressed as means of three replicates' determinations (n = 3) ± standard deviation (SD).

Results

Identification and screening of biocontrol fungi and bacteria for antagonistic activity against gray blight disease

Twenty-eight fungal and 32 bacterial strains were collected from deep-sea sediment samples. The isolates were screened for antagonistic effect against *P. theae,* using the plate confrontation method. Isolate A65 suppressed the growth of the test fungus *P. theae* by 64.34% and showed a zone of inhibition of 15.08 mm (Fig. 1). Isolate B5 clearly suppressed the growth of the test fungus *P. theae* by 72% and showed a zone of inhibition of 15.45 mm (Fig. 1). Isolates A65 and B5 were selected for further evaluation of their biocontrol potential against gray blight disease under micro-plot field conditions.

Isolate A65 showed a rapid growth on potato dextrose agar medium that contained 2% NaCl. Analysis of the microbiological characteristics suggested that the fungus A65 grew rapidly on potato dextrose agar plates, the colony color was light purple after 3 days and was protruding with a powdery surface and colorless mycelium. The internal transcribed spacer sequence of A65 was generated for 620 nucleotide base pairs (Additional file 1: Fig. S1). Sequence analysis showed the closest match (99% similarity) with *Paecilomyces lilacinus* strain B59A (GenBank ID: HM242263.1), demonstrating that strain A65 was a typical member of the genus *Paecilomyces*. Based on these results, isolate A65 was designated as *P. lilacinus* A65. The strain was deposited in the China Center for Type Culture Collection (CCTCC) under accession number M2012384 (CCTCC-M2012385). The gene sequences were also submitted to GenBank under accession number JX978452.1.

Isolate B5 grew well in Luria–Bertani medium that contained 2% NaCl. Light microscopy showed that this bacterium was rod-shaped and gram-positive. Pale-yellow colonies with irregular and opaque edges were observed on the culture plate. Over time, the colony became folded, and the middle of the fold was raised. The 16S rRNA sequence of B5 was generated for 1,511 base pairs (Additional file 1: Fig. S2). Sequence analysis showed the closest match (99% similarity) with *Bacillus subtilis* NCIB 3610, demonstrating that strain B5 was a typical member of the genus *Bacillus*. Isolate B5 was designated as *B. subtilis* B5 based on these results. The strain was deposited in the CCTCC under the accession number M2012384 (CCTCC-M2012384). The gene sequence was also submitted to GenBank under the accession number JX978451.1.

Screening of A65 and B5 for antagonistic activity against different fungal pathogens of tea

The inhibition zones caused by strains A65 and B5 ranged (from 1 to 6 mm) and (3–8 mm), respectively. The pathogenic species showed varying sensitivities to the antagonistic effects of A65 and B5. The inhibition of A65 ranged from 52 to 64%, whereas that of B5 ranged from 70 to 76%, as calculated using Eqs. (2) and (3) (Table 2).

Formula of wettable powder

The composition of the biological control agent A65 wettable powder was details as follows: 1% PEG800, 5% tea saponin, 0.5% $CaCO_3$, 82.5% diatomite, and 10% spore and A65. Subsequently, 1 g of wettable powder was added to the suspension. The suspension was diluted by 100-, 500-, and 1000-fold (bacterial contents of 1×10^8, 2×10^7, and 1×10^7 colony-forming units (CFU)/ml, respectively) for field trials.

The composition of the biological control agent B5 wettable powder was 5% Morwet EFW, 3% Morwet

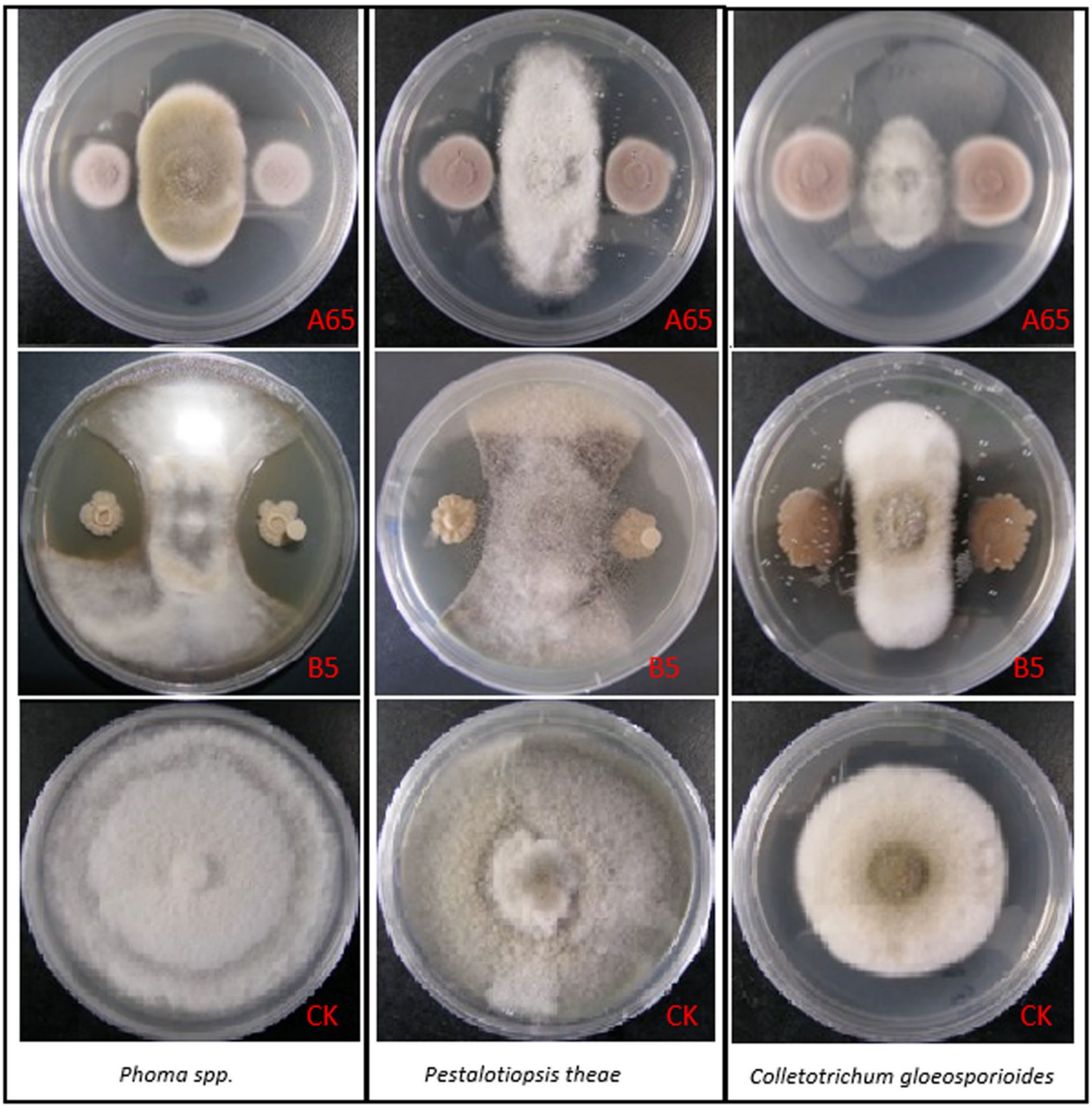

Fig. 1 Inhibition effects of B5/A65 on the pathogenic fungi of tea

D-425, 0.5% sodium carboxymethyl cellulose, 0.3% sodium fluorescein, and supplemented to 100% with precipitate powder (B5 cells and diatomite). The wettable powder (1 g) was added to the suspension, diluted by 100-, 500-, and 1000-fold (bacterial contents of 1×10^8, 2×10^7, and 1×10^7 CFU/ml, respectively) for field trials.

Efficacy of a biocontrol agent as wettable powders against the gray blight disease of tea in field trials

Effective antagonists A65 and B5 were evaluated to assess their efficacy for controlling gray blight disease in a micro-plot field trial. The inhibition ratio was determined after the first and second sprays. Among the various treatments, plots sprayed with A65 (10^8 CFU/ml) showed

Table 2 Inhibition effects of biocontrol agent A65 and B5 on the pathogenic fungi of tea

Plant pathogens	Inhibition zones (mm)		Inhibition (%)	
	B5	A65	B5	A65
Phoma sp. L2	8.38 ± 0.34	3.78 ± 0.32	70.42	57.34
Pestalotiopsis theae LH13	2.60 ± 0.08	4.32 ± 0.37	72.53	64.34
Colletotrichum gloeosporioides LH30	5.08 ± 0.30	6.30 ± 0.54	71.73	63.93
Neofusicoccum sp. LH107	4.26 ± 0.33	1.68 ± 0.06	74.53	53.32
Alternaria sp. LH129	7.32 ± 0.44	1.52 ± 0.10	76.28	52.20
Pestalotiopsis sp. LH162	3.20 ± 0.13	2.04 ± 0.09	70.32	63.94

higher inhibition ratio than those of other treatments, followed by chemical fungicide treatment. Compared to the untreated control, A65 and chemical treatment inhibited gray blight disease by 75.46 and 76.34% after the first and second sprays, respectively (Table 3). Additionally, plots sprayed with B5 (10^8 CFU/ml) showed higher inhibition ratio than those of the other treatments, followed by chemical fungicide treatment. Compared to the untreated control, B5 and chemical treatment inhibited gray blight disease by 78.57 and 76.34% after the first and second sprays, respectively (Table 3). These results indicated that the biocontrol agent A65 and B5 controlled gray blight disease at similar rates as chemical fungicides.

Discussion

Deep-sea microorganisms have many unique functions that differ from those of terrigenous and shallow-sea microorganisms because of their habitats (Zhu et al. 2017). They have also gained attention because of their potential applications (Mo et al. 2022). New research techniques have recently been applied to accelerate the research and development of deep-sea microorganism resources (Hu et al. 2020). Deep-sea microorganisms can produce numerous secondary metabolites with novel structures, unique functions, and low toxicity because they have adapted to living in extreme deep-sea environments, such as having high pressure (Yu et al. 2022), low temperature (e.g., hydrothermal vents) (Bo et al. 2022), anaerobic (Bo et al. 2022), extreme pH gradient (Yu et al. 2022), high salt concentration, (Khouadja et al. 2014) high metal ion concentration, dark (Merlino et al. 2018), oligotrophic (Xia et al. 2021), and high halogen concentration (Cui et al. 2020) conditions. The antifungal activities of deep-sea fungi have become a hotspot of research and development studies (Sun et al. 2020).

Screening against a wide range of fungal pathogens of tea showed that A65 and B5 effectively inhibited the mycelial growth of *P. theae* L13 (Nagata et al. 1992), *C. gloeosporioides* L30 (Rabha et al. 2016), *Phoma* sp. L2 (Zhao et al. 2018), *Neofusicoccum* sp. L107 (Ploetz et al. 2009), *Alternaria* sp. L129 (Kirubakaran and Sakthivel 2007), and *Pestalotiopsis* sp. L162 (Palanisamy and Mandal 2014).

P. lilacinus has been reported in several countries and regions worldwide and has been used for the biocontrol of the root-rot disease complex of chickpea caused by *Meloidogyne incognita* race and *Macrophomina phaseolina*. (Shafique et al. 2015) The biocontrol agent *P. lilacinus* can colonize the eggs of *Meloidogyne* spp. The antagonistic characteristics of this biocontrol agent and the combination for controlling *M. incognita* were investigated (Wang et al. 2010). The potential of *B. subtilis* as a biofungicide for controlling fungal diseases in plants under field and in vitro conditions has been reported. Commercially acceptable formulations of *B. subtilis* CPA-8 have a long storage life and retain their efficacy when spray-dried for controlling peach and nectarine brown rot caused by *Monilinia* sp. (Yánez Mendizábal et al. 2012a; b). An antagonistic *B. subtilis* isolate strongly inhibited the growth of *Cercospora lactucae-sativae* leaf

Table 3 Effects of antagonistic microorganism on gray blight dise ase incidence in tea plants under micro-plot

Groups	Untreated disease index (%)	Testing after first spray		Untreated disease index (%)	Testing after second spray		Inhibition average ratio (%)
		Disease index (%)	Inhibition ratio (%)		Disease index (%)	Inhibition ratio (%)	
1	3.35	5.87	74.42	5.87	6.08	76.51	75.46
2	4.81	7.21	68.58	7.21	8.17	68.43	68.51
3	3.62	7.04	69.32	7.04	8.33	67.81	68.57
4	3.98	4.64	79.78	4.64	5.86	77.36	78.57
5	3.65	5.12	77.69	5.12	6.67	74.23	75.96
6	5.02	5.95	74.07	5.95	7.04	72.80	73.44
7	4.66	5.20	77.34	5.20	6.38	75.35	76.34
8	4.17	22.95	–	22.95	25.88	–	–

spots at an inhibition rate of 80.82% (Sehsah et al. 2022). 7-*O*-2′*E*-Butenoyl macrolactin A and macrolactin A were isolated from *B. subtilis* and exhibited antifungal activity against the tea pathogenic fungi *P. theae* and *C. gloeosporioides* (Li et al. 2016). Wang found a linear positive relationship between the mycelial growth rate of *Pestalotiopsis* sp. and pathogenicity; strains with fast mycelium growth exhibit high pathogenicity (Wang et al. 2019a, b).

Bacillus subtilis is considered to colonize tea plants, occupy favorable niches, and alter the ecological function of microbial communities in plants, thereby inhibiting pathogen infection and development (Pandey and Palni 1997). Additionally, *B. subtilis* and *P. lilacinus* can produce antimicrobial proteins or other active substances that inhibit the mycelial growth of fungus and formation and germination of conidia, thereby killing the pathogens or inhibiting their growth and development (Wang et al. 2019a, b). *B. subtilis* and *P. lilacinus* may also induce the formation of reactive oxygen species, reduce the lipid peroxidation of membranes, and induce the production of related enzymes, such as phenylalaninammo-nialyase (PAL) and β-1, 3-glucoamylase in tea plants. The corresponding disease-related proteins in tea plants can kill or inhibit the development of pathogenic bacteria (Wang et al. 2021).

Wettable powder is among the four major pesticides developed and have been widely used to prevent and control crop diseases. In recent years, new auxiliaries and carriers have been applied in preparing wettable powders, which have played important roles in agricultural production. As such, a formulation of wettable fungicides that contain two active strains namely B5 and A65 was proposed. The fungicides showed a good wettability, suspension, storage stability, and other performance indicators in field experiments.

Isolates *P. lilacinus* A65 and *B. subtilis* B5 inhibited *P. theae* L13 by 75.46 and 72% on potato dextrose agar medium, respectively. Isolates of A65 and B5 also exhibited apparent antagonistic activity against various pathogens in tea, thereby demonstrating their potential as a biological agent. Notably, A65 and B5 only showed strong inhibitory effects when they were inoculated at the same time as or before the inoculation of pathogenic bacteria, hence showing that they could only prevent infection by pathogenic fungi and could not kill the fungi after the occurrence of the disease. However, the application of these biological pesticides can prevent the formation of plant disease spots and reduce economic losses of tea. Further studies should focus on the soil colonization ability, toxicity, environmental toxicity, environmental residues and other indicators of B5 and A65, as well as the mechanism of interaction among these isolates and the environment.

Conclusion

Two biocontrol agents, namely, *P. lilacinus* A65 and *B. subtilis* B5 were developed. In vitro and field experiments showed that the biocontrol agents controlled gray blight disease caused by *P. theae* in tea. The use of these microorganisms to control fungal plant pathogens is lesser costly than the use of synthetic fungicides.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41938-023-00701-3.

Additional file 1: Figure S1. ITS sequence of strain A65. **Figure S2.** 16S rRNA sequence of strain B5.

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Author contributions
XT designed the field plot experiment and guided the work throughout; FY analyzed and interpreted the data regarding the Field Trials. GX performed activity verification and strain screening and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated during and/or analyses during the current study are available in the NCBI—National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). All data included in this study are available upon request by contacting the corresponding author.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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