Rhizosphere 38 (2026) 101382

Contents lists available at ScienceDirect

Rhizosphere

journal homepage: www.elsevier.com/locate/rhisph

Heat-stable metabolites from a *Bacillus* strain with broad-spectrum antifungal activity are effective against *Rhizoctonia solani* in rice

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ARTICLE INFO

Keywords:
Antimycotic
Crop pathogen
Mycolytic activity
Plant immunity
Rhizobacteria

ABSTRACT

Rice sheath blight, caused by *Rhizoctonia solani*, is a major constraint to sustainable rice production, yet mechanistic understanding of effective biological control remains limited. In this study, a mycolytic bacterial strain, *Bacillus cereus* strain KT-9, was evaluated for its antifungal activity and underlying mechanisms. Strain KT-9 completely inhibited *R. solani* growth in dual culture assays (100% inhibition) and significantly suppressed fungal growth in culture filtrates, with 93.4% inhibition at 70% (v/v), demonstrating broad-spectrum antifungal activity. Its culture filtrates showed activity comparable to prochloraz and higher than azoxystrobin, while retaining full activity after autoclaving and suppressing sclerotial germination, indicating the presence of heat-stable bioactive metabolites. LC–QTOF–MS analysis revealed diverse putatively identified metabolites, including cyclic peptides, peptide-derived metabolites, aromatic acid derivatives, and maculosin, which may contribute to the observed antifungal activity. Exposure of *R. solani* to strain KT-9 culture filtrates induced oxidative stress, as indicated by increased reactive oxygen species levels, elevated antioxidant enzyme activities, and disruption of glutathione redox balance. In greenhouse experiments, strain KT-9 treatments enhanced rice growth, increasing plant height by 57% (33.8 cm vs. 21.5 cm in control) and reducing disease severity by 55% compared with pathogen-inoculated controls. Overall, these findings suggest that strain KT-9 suppresses rice sheath blight through multiple interacting mechanisms, including production of heat-stable antifungal metabolites, induction of oxidative stress in the pathogen, and direct mycolytic activity. This study highlights the potential of *B. cereus* strain KT-9 as a sustainable biocontrol agent and provides insight into its antifungal mechanisms.

1. Introduction

Rice sheath blight caused by *Rhizoctonia solani* is one of the most destructive diseases affecting rice production worldwide, leading to substantial yield losses under favorable environmental conditions (Li et al., 2021a; Abbas et al., 2023; Gupta and Gaur, 2024). Current management strategies rely heavily on synthetic fungicides; however, their long-term application is constrained by environmental concerns, including environmental persistence of chemical residues, adverse non-target effects on beneficial soil and microbial communities, and the development of pathogen resistance through continuous selection pressure, as well as inconsistent efficacy under field conditions (Faria-Ramos et al., 2014; Ou et al., 2025). These limitations have driven increasing interest in sustainable alternatives, particularly biological control using beneficial microorganisms.

Among biocontrol agents, plant growth-promoting rhizobacteria (PGPR), especially *Bacillus* spp., are widely recognized for their ability to suppress phytopathogenic fungi and enhance plant growth (Ali et al., 2022; Etesami et al., 2023; de Andrade et al., 2023). The antagonistic activity of these bacteria is commonly attributed to the production of diverse antifungal metabolites, including lipopeptides, polyketides, and other secondary metabolites (Ongena and Jacques, 2008; Chen et al., 2009; Caulier et al., 2019; Markelova and Chumak, 2025; Dutilloy et al., 2026). Several studies have demonstrated the effectiveness of

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https://doi.org/10.1016/j.rhisph.2026.101382
Received 24 March 2026; Received in revised form 14 May 2026; Accepted 14 May 2026
Available online 16 May 2026

Bacillus-based biocontrol agents in reducing sheath blight severity in rice (Kumar et al., 2012; Shrestha et al., 2016; Tao et al., 2024; Ansari et al., 2025; Krishnan et al., 2025). Nevertheless, most studies have primarily focused on phenotypic suppression, while the underlying biochemical and physiological mechanisms remain insufficiently explored.

Recent advances in metabolomic approaches provide new opportunities to elucidate the chemical basis of microbial antagonism, enabling the identification of bioactive compounds involved in pathogen inhibition (Nguyen et al., 2025; Villavicencio-Vásquez et al., 2025). In particular, heat-stable antifungal metabolites have attracted attention due to their stability and applicability under diverse environmental conditions, as demonstrated in several microbial systems (Boukaew and Prasertsan, 2014; Abdelmoteleb et al., 2023). Emerging evidence also suggests that induction of oxidative stress is a key mechanism in microbial antagonism, whereby reactive oxygen species (ROS) accumulation leads to cellular damage and inhibits the growth of fungal pathogens (Yaakoub et al., 2022; Park and Son, 2024). Similar oxidative stress–mediated antifungal effects have been reported in *Bacillus*-derived metabolites (Zhao et al., 2022; Fan et al., 2023). However, integration of metabolomic evidence with functional assays to reveal multi-mechanistic antifungal strategies remains limited.

In this study, a mycolytic bacterial strain identified as *B. cereus* strain KT-9 was investigated for its antifungal activity against *R. solani*. We hypothesized that strain KT-9 may suppress rice sheath blight through multiple mechanisms, including the production of antifungal metabolites and oxidative stress–mediated inhibition of fungal growth. To test this hypothesis, we aimed to investigate the potential antifungal mechanisms underlying the biocontrol efficacy of strain KT-9 against *R. solani*.

2. Material and methods

2.1. Fungal pathogens and culture conditions

Rhizoctonia solani (AG-1 IA), isolated from infected rice plants, was obtained from the Phatthalung Rice Research Center (Thailand) and maintained on potato dextrose agar (PDA; HiMedia™) at 28 ± 2 °C. Sclerotia were produced by incubation for 10 days and used for subsequent experiments. For disease evaluation under greenhouse conditions, inoculum was prepared using colonized wheat seeds as previously described (Park et al., 2008; Tsai et al., 2012).

Additional phytopathogenic fungi used for broad-spectrum antifungal evaluation, including *Schizophyllum commune* Fr. (SOPRC-07), *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (NM-01), *Peniophora salaccae* Boukaew et al. (2024) (SKRU002), *Curvularia oryzae* Bugnicourt (SOPRC-9), *Aspergillus flavus* Link (PSRDC-4), *Aspergillus parasiticus* Speare (TISTR 3276), *Sclerotium rolfsii* Sacc. (KKU 01), *Corynespora cassiicola* (Berk. & M.A. Curtis) C.T. Wei (PSU-01), and *Fusarium incarnatum* (Desm.) Sacc. (PSU-01), were obtained from established culture collections and maintained on PDA under standard laboratory conditions.

2.2. Soil sample collection and bacterial isolation

Rhizosphere soil samples were collected from five rice-growing sites in Ko Taeo Subdistrict (7.092140° N, 100.622502° E), Mueang Songkhla District, Songkhla Province, Thailand. The climate is classified as tropical monsoon **(**Köppen–Geiger climate classification: Am**)**, characterized by warm temperatures throughout the year (annual average ~27–28 °C) and high annual rainfall (~1900–2000 mm) (Peel et al., 2007). Bacterial isolation was performed using a standard serial dilution plating method as previously described (Boukaew et al., 2011). Briefly, soil suspensions were prepared in sterile saline solution (0.85% NaCl), serially diluted, and spread onto nutrient agar (NA; HiMedia™), followed by incubation at 28 ± 2 °C. Morphologically distinct colonies were selected, purified by repeated streaking, and maintained on NA at 4 °C for further use.

2.3. Screening of antifungal activity

Antagonistic activity of bacterial isolates against *R. solani* was evaluated using both direct (dual culture) and indirect (volatile-mediated) assays.

2.3.1. Dual culture assay

Antifungal activity was assessed using a dual culture assay as described by Mannaa et al. (2025). Briefly, bacterial isolates were streaked onto NA and pre-incubated to allow colony establishment. A mycelial plug (5 mm diameter) from an actively growing *R. solani* culture was then placed at a fixed distance from the bacterial colony. Plates inoculated with *R. solani* alone served as controls. After incubation at 28 ± 2 °C for 3 days, radial growth of *R. solani* was measured. The percentage of radial growth inhibition was calculated as follows: inhibition (%) = [(D1 − D2)/D1] × 100, where D1 represents the radial growth of the fungal pathogen in the control treatment and D2 represents the radial growth in the presence of bacterial isolates. All experiments were conducted with three replicates and repeated twice.

2.3.2. Volatile organic compounds (VOC) assay

The effect of bacterial VOC on the mycelial growth of *R. solani* was evaluated using a sealed plate method (Calvo et al., 2020). Briefly, bacterial isolates were cultured on NA plates, while *R. solani* was inoculated onto PDA. The two plates were paired face-to-face and sealed to permit VOC-mediated interaction without physical contact (Fig. S1, Supplementary data). Plates containing only *R. solani* served as controls. After incubation at 28 ± 2 °C for 3 days, fungal growth was measured, and inhibition percentage was calculated as described above. All experiments were conducted with three replicates and repeated twice.

2.4. Genome analysis and biosynthetic gene clusters

Whole-genome sequencing of strain KT-9 was performed using an Illumina platform. Raw reads were quality-filtered and *de novo* assembled using Unicycler (Wick et al., 2017). Genome annotation was conducted using PROKKA to predict coding sequences and structural RNA genes (Seemann, 2014). Phylogenomic analysis was performed using the AutoMLST2 pipeline with publicly available genomes of closely related *Bacillus* species. Pairwise genome similarity was estimated based on average nucleotide identity (ANI), and conserved single-copy marker genes were used to construct a concatenated alignment. A maximum-likelihood phylogenomic tree was inferred using IQ-TREE with 1000 bootstrap replicates. Biosynthetic gene clusters associated with secondary metabolite production were predicted using antiSMASH (v6.0.1) and compared with reference clusters in the MIBiG database to assess similarity and potential functional annotation.

2.5. Antifungal activity of culture filtrates

The antifungal activity of culture filtrates of strain KT-9 against *R. solani* was evaluated in both solid (PDA) and liquid (potato dextrose broth, PDB; HiMedia™) media. Briefly, strain KT-9 was cultured in nutrient broth, and cell-free culture filtrates were obtained by centrifugation followed by membrane filtration (0.2 μm). For the PDA assay, culture filtrates were incorporated into PDA at different concentrations (10–70%, v/v). A mycelial plug (5 mm diameter) from an actively growing *R. solani* culture was placed at the center of each plate, and fungal growth was measured after incubation at 28 ± 2 °C. For the PDB assay, culture filtrates were added to liquid medium at the same concentrations, followed by inoculation with *R. solani*. After incubation, fungal biomass was determined gravimetrically, and growth inhibition was calculated as described above. All experiments were conducted with three replicates and repeated twice.

2.6. Broad-spectrum antifungal activity

The broad-spectrum antifungal activity of strain KT-9 culture filtrates was evaluated against multiple phytopathogenic fungi. Culture filtrates (60%, v/v) were incorporated into PDB, followed by inoculation with different fungal pathogens. After incubation under appropriate conditions depending on fungal growth rates, mycelial biomass was determined, and growth inhibition was calculated as described above. All experiments were conducted with three replicates and repeated twice.

2.7. Comparison of culture filtrates with synthetic fungicides

The antifungal efficacy of strain KT-9 culture filtrates (60%, v/v) was compared with selected commercial synthetic fungicides, including prochloraz, azoxystrobin, and propiconazole, at different concentrations (0.1–1.0%, v/v), following previously described methods for antifungal bioassays (Petlamul et al., 2026). These synthetic fungicides were selected as representative fungicides commonly used for rice disease management and commercially available in Thailand. Treatments were incorporated into PDA, followed by inoculation with a mycelial plug (5 mm diameter) from an actively growing *R. solani* culture. Plates without culture filtrates or synthetic fungicides served as controls. After incubation at 28 ± 2 °C for 3 days, fungal growth was measured, and inhibition percentage was calculated as described above. All experiments were conducted with three replicates and repeated twice.

2.8. Heat and dilution stability of antifungal activity

The stability of antifungal activity in strain KT-9 culture filtrates was evaluated under heat treatment and serial dilution conditions. Culture filtrates were either used directly, diluted with PDB (1:10 to 1:1,000, v/v), or subjected to autoclaving (121 °C, 15 min). Aliquots (1 mL) of each treatment were transferred into 24-well plates, and five uniformly sized sclerotia of *R. solani* were added to each well. Sclerotial germination percentages were recorded after 24 and 48 h. All experiments were conducted with three replicates and repeated twice.

2.9. Identification of bioactive metabolites by LC–QTOF–MS

Bioactive metabolites in the culture filtrates of strain KT-9 were analyzed using liquid chromatography–quadrupole time-of-flight mass spectrometry (LC–QTOF–MS) on an Agilent 1290 Infinity II UHPLC system coupled to a 6545 Q-TOF mass spectrometer (Agilent Technologies, USA). Sample preparation followed previously described protocols. Chromatographic separation was performed on a C18 column using a water–methanol gradient containing 0.1% acetic acid at a constant flow rate. Mass spectrometric analysis was conducted in both positive and negative electrospray ionization modes with data-dependent MS/MS acquisition. Putative identification of metabolites was achieved by comparing accurate mass measurements and MS/MS fragmentation patterns with entries in the METLIN database using MassHunter software.

2.10. Screening of plant growth–promoting traits

2.10.1. In vitro screening of plant growth–promoting traits

The strain KT-9 was screened for plant growth–promoting (PGP) traits, including indole-3-acetic acid (IAA) production, ammonia production, siderophore production, and the solubilization of zinc and phosphate. IAA production was determined colorimetrically in the presence of L-tryptophan following the method of Glickmann and Dessaux (1995). Phosphate and zinc solubilization were evaluated on selective media following the methods of Pande et al. (2017) and Gandhi and Muralidharan (2016), respectively, and expressed as solubilization indices. Siderophore production was assessed using chrome azurol S agar according to Schwyn and Neilands (1987), while ammonia production was determined using Nessler's reagent following Cappuccino and Sherman (2001). All experiments were conducted with three replicates and repeated twice.

2.10.2. Effect of strain KT-9 on rice growth

The plant growth–promoting effect of strain KT-9 was evaluated under greenhouse conditions. Rice seeds were treated with (i) sterile distilled water (control), (ii) bacterial suspension (1×10^7 colony-forming units (CFU)/mL), (iii) culture broth, or (iv) culture filtrates, and subsequently grown in sterilized soil. Treatments were applied weekly for four weeks, and plant height was measured one week after the final application. Each treatment consisted of three replicates (n = 18 pots per treatment).

2.11. Evaluation of strain KT-9 against rice sheath blight disease

The biocontrol efficacy of strain KT-9 against rice sheath blight caused by *R. solani* was evaluated under greenhouse conditions using a completely randomized design. Treatments included uninoculated and untreated plants (negative control), inoculated plants treated with sterile distilled water (positive control), bacterial suspension of strain KT-9 (1×10^7 CFU/mL), strain KT-9 culture broth, strain KT-9 culture filtrates, prochloraz (1.0%, v/v), and propiconazole (1.0%, v/v). Plants were inoculated with *R. solani* using colonized substrate following the method of Park et al. (2008). Disease severity was assessed using a 0–9 rating scale according to the International Rice Research Institute (2013). Disease index and control efficacy (CE) were calculated accordingly. Each treatment consisted of three replicates (n = 18 pots per treatment).

2.12. Mycolytic activity and antifungal mechanisms of strain KT-9

2.12.1. Mycolytic activity assay

The ability of strain KT-9 to utilize mycelial biomass of *R. solani* as a nutrient source was evaluated using a modified mycolytic activity assay based on Mannaa et al. (2023). Briefly, strain KT-9 was co-incubated with fungal mycelial biomass under nutrient-limited conditions, and bacterial growth was quantified by CFU enumeration. Treatments without fungal biomass served as controls. All experiments were conducted with three replicates and repeated twice.

2.12.2. Antifungal mechanism analysis

To investigate oxidative stress–related mechanisms underlying the antifungal activity of strain KT-9 culture filtrates, intracellular reactive oxygen species (ROS), antioxidant enzyme activities, and glutathione redox status in *R. solani* were analyzed. Fungal biomass was exposed to culture filtrates under controlled conditions. ROS levels were determined using the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) fluorescent probe (Keston and Brandt, 1965), antioxidant enzyme activities including catalase (CAT) and superoxide dismutase (SOD) (Beers and Sizer, 1952; Kostyuk and Potapovich, 1989), and glutathione redox status (GSH/GSSG) (Kostyuk and Potapovich, 1989). Total protein content was quantified using the Lowry method (Lowry et al., 1951). These parameters served as indicators of oxidative stress induction, cellular redox imbalance, and disruption of the fungal antioxidant defense system (Yaakoub et al., 2022; Park and Son, 2024). All experiments were conducted with three replicates and repeated twice.

2.13. Statistical analysis

All experimental data were analyzed using IBM SPSS Statistics 26 (IBM Corp., Armonk, NY, USA). Data were tested for normality and homogeneity of variance prior to analysis. One-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference test, was used to evaluate differences among multiple treatments at $p < 0.05$. For

pairwise comparisons between the treated and untreated control groups, independent-samples t-tests were performed at $p < 0.05$. Asterisks indicate significant differences between the treated and untreated control groups.

3. Results

3.1. Screening of antifungal activity

Thirty bacterial isolates obtained from rhizosphere soil were screened for antifungal activity against *R. solani* using dual culture and VOC assays (Fig. S2 and Table S1, Supplementary data). In the dual culture assay, all isolates significantly inhibited *R. solani* mycelial growth ($p < 0.05$), with inhibition ranging from 49.8% to 100%. Five strains (strain KT-9, strain KT-27, strain KT-29, strain KT-34, and strain KT-36) exhibited strong antifungal activity (>70%), with strain KT-9 completely inhibiting fungal growth (100%). The remaining isolates showed moderate activity (50.0–69.4%) (Fig. S2A).

Twelve isolates with >65% inhibition were further evaluated for VOC-mediated suppression in sealed plate assays. VOC effectiveness ranged from 0 to 80.4%, with strain KT-21 showing the highest suppression (80.4%) and strain KT-6 also above 66% (Fig. S2B). Based on its strong and consistent activity in both assays, strain KT-9 was selected for detailed studies.

3.2. Genome analysis and biosynthetic gene clusters

Whole-genome sequencing of strain KT-9 yielded a single circular chromosome of 5,401,338 bp with 37.75% GC content (Fig. 1; Table S2, Supplementary data). Genome annotation predicted 5678 protein-coding sequences, 58 tRNA genes, and 4 rRNA genes. Phylogenomic analysis placed strain KT-9 within the *Bacillus cereus* clade, forming a well-supported monophyletic cluster with *B. cereus* GCF_000007825 (Fig. S3, Supplementary data), confirming its taxonomic identity.

Genome mining identified 21 putative biosynthetic gene clusters for secondary metabolites, including terpenes, non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), ribosomally synthesized and post-translationally modified peptides (RiPPs), siderophores, and beta-lactones (Table S3). Several clusters matched known metabolites, such as sodorifen, a volatile compound associated with microbial interactions, and the siderophores bacillibactin and petrobactin, which are involved in iron acquisition and microbial competition, potentially contributing to the strong antifungal activity observed. One RiPP-like cluster showed no similarity to known biosynthetic gene clusters, suggesting a potentially novel metabolite.

3.3. Antifungal activity of culture filtrates

The antifungal activity of *B. cereus* strain KT-9 culture filtrates against *R. solani* showed a clear dose-dependent pattern in both PDA and PDB media (Table S4, Supplementary data). On PDA (Table S4A), mycelial growth inhibition increased from 35.18% at 45% culture filtrates to 71.90% at 60% culture filtrates, reaching 93.36% at 70% culture filtrates, indicating near-complete suppression. In PDB (Table S4B), inhibition was stronger at lower concentrations; 30% culture filtrates reduced fungal growth by 69.07%, over 4.5-fold higher than PDA at the same concentration (15.49%). At higher concentrations (45–70% culture filtrates), inhibition in PDB reached 94.62–95.53%, with no significant differences among treatments ($p > 0.05$). These results indicate that strain KT-9 metabolites are highly effective, especially in liquid medium, and their antifungal activity increases with concentration.

3.4. Broad-spectrum antifungal activity

The culture filtrates of *B. cereus* strain KT-9 exhibited significant antifungal activity ($p < 0.05$) against nine phytopathogenic fungi, indicating the production of diffusible metabolites with broad-spectrum effects (Fig. 2). Comparative analysis (Fig. 2A and B) showed mycelial growth inhibition ranging from 8.43% to 62.03%. The strongest inhibition was observed against *L. theobromae* (62.03%), closely followed by *S. commune* (60.78%), while *P. salaccae* was the least sensitive (8.43%). These findings confirm that strain KT-9 produces bioactive metabolites capable of suppressing a diverse array of fungal pathogens, supporting its potential as a biocontrol agent for various plant diseases.

3.5. Comparison of culture filtrates with synthetic fungicides

The antifungal activity of *B. cereus* strain KT-9 culture filtrates (70%

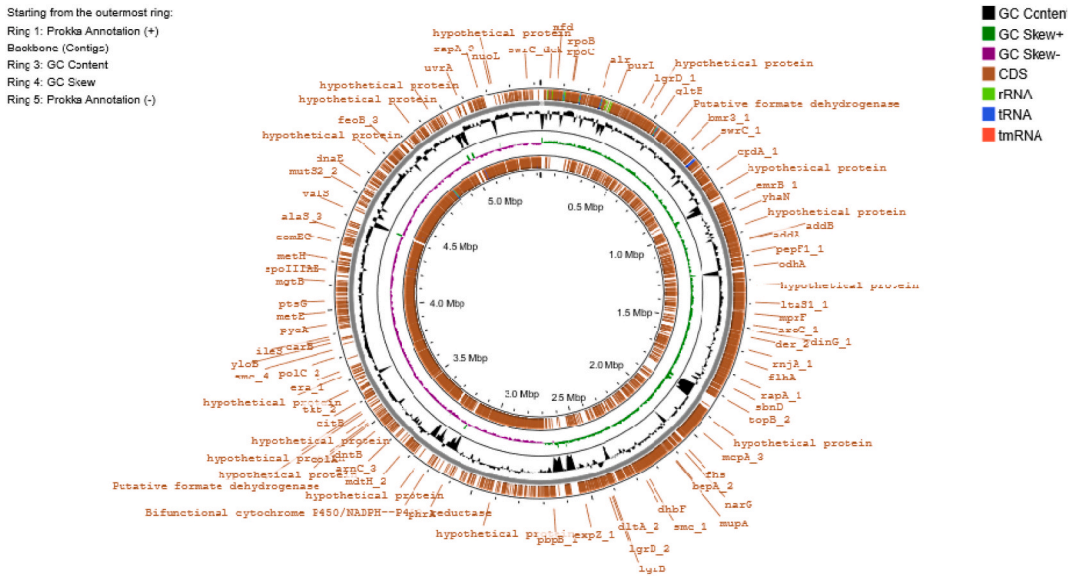

Fig. 1. Circular genome map of *B. cereus* strain KT-9 generated through Proksee web-based tool. The tracks display open reading frames (ORFs) on forward and reverse strands, backbone contigs, GC content, and GC skew (green/purple graph), illustrating the detailed genomic structure. The outermost ring shows ORFs on the positive strand, followed by backbone contigs. The third ring represents GC content with black peaks for higher GC areas, and the fourth ring shows GC skew, with green for positive (GC Skew+) and purple for negative (GC Skew-). The innermost ring shows ORFs on the negative strand.

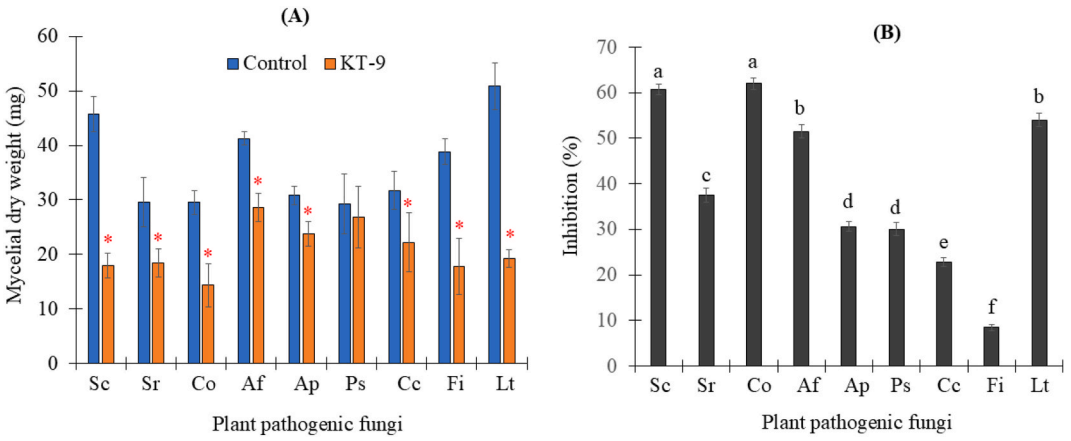

Fig. 2. Broad-spectrum antifungal activity of *B. cereus* strain KT-9 culture filtrates against *S. commune* (Sc), *S. rolfsii* (Sr), *C. oryzae* (Co), *A. flavus* (Af), *A. parasiticus* (Ap), *P. salaccae* (Ps), *C. cassiicola* (Cc), *F. incarnatum* (Fi), and *L. theobromae* (Lt) cultured in PDB at 28 ± 2 °C for 3–7 days. **(A)** Mycelial growth inhibition of each fungal isolate treated with strain KT-9 culture filtrates. Asterisks indicate significant differences between treated and control groups for each fungal isolate (*$p < 0.05$). **(B)** Percentage of growth inhibition among different fungal isolates. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey's HSD test. Data are expressed as the mean ± SD (n = 3).

v/v) was compared with prochloraz, azoxystrobin, and propiconazole at 0.1–1.0% (v/v) (Fig. 3). Strain KT-9 culture filtrates strongly inhibited *R. solani* growth (93.36%), significantly ($p < 0.05$) higher than prochloraz at 0.1% (78.76%) and 0.2% (88.91%), but lower than prochloraz at ≥0.4%. Azoxystrobin showed moderate inhibition (32.41–56.30%) across concentrations, consistently lower than strain KT-9 culture filtrates. Propiconazole was the most effective, achieving complete inhibition (100%) at all tested concentrations. Overall, strain KT-9 culture filtrates demonstrated antifungal activity comparable to prochloraz at 0.2% and substantially higher than azoxystrobin, highlighting its potential as a biocontrol alternative.

3.6. Heat- and dilution-stability of antifungal activity

The effect of *B. cereus* strain KT-9 culture filtrates on *R. solani* sclerotial germination was significant ($p < 0.05$) (Fig. S4, Supplementary data). Undiluted culture filtrates completely inhibited germination at 24 and 48 h. Dilution gradually reduced efficacy: at 1:10, germination remained 0% at 24 h but rose to 66.7% at 48 h; at 1:100, 0% at 24 h and 83.3% at 48 h; at 1:1,000, 66.7% at 24 h and 100% at 48 h, similar to the control. Autoclaved culture filtrates (121 °C, 15 min) fully suppressed germination at both time points, indicating heat-stable antifungal compounds.

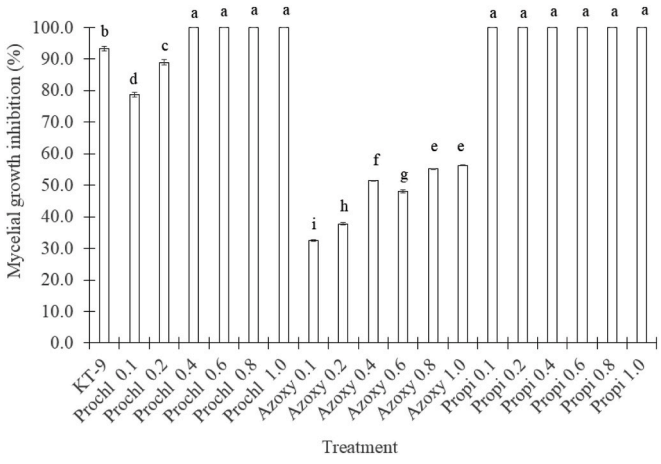

Fig. 3. Comparative antifungal activity of *B. cereus* strain KT-9 culture filtrates at a concentration of 70% (v/v) and three synthetic fungicides (prochloraz, azoxystrobin, and propiconazole) at concentrations of 0.1–1.0% (v/v) against the mycelial growth of *R. solani* on PDA after 3 days of incubation at 28 ± 2 °C. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey's HSD test. Data are expressed as the mean ± SD (n = 3). Abbreviations: Prochl, prochloraz; Azoxy, azoxystrobin; Propi, propiconazole.

3.7. Identification of bioactive metabolites by LC–QTOF–MS

LC–QTOF–MS analysis of culture filtrates from *B. cereus* strain KT-9 detected multiple putatively identified metabolites in both negative and positive ionization modes, including peptides, cyclic peptides, amino acid derivatives, organic acids, aromatic compounds, and lipid-related metabolites (Tables S5 and S6, Supplementary data). In negative ionization mode (Table S5), several metabolites previously reported to possess antimicrobial or antifungal-related properties were detected, including hydroxyphenyllactic acid, benzoic acid, trans-cinnamic acid, indolelactic acid, anthranilic acid, and maculosin, together with amino acid–derived compounds and diketopiperazine-related metabolites such as cyclo-Ala-Pro diketopiperazine. In positive ionization mode (Table S6), various oligopeptides and cyclic peptide-related compounds were identified, including cyclo(alanylvalyl), L,L-cyclo(leucylprolyl), maculosin, aspergillic acid, and several amino acid-containing peptides. Maculosin was consistently detected in both ionization modes, suggesting that it may represent one of the prominent metabolites produced by strain KT-9 under the tested conditions. In addition, aromatic acid derivatives and peptide-related metabolites detected in both modes may contribute to the antifungal activity observed in the bioassays. Overall, the LC–QTOF–MS profiles indicate that strain KT-9 produces diverse secondary metabolites potentially associated with antifungal activity.

3.8. Screening of plant growth-promoting traits and their effect on rice growth

B. cereus strain KT-9 exhibited multiple plant growth–promoting (PGP) activities (Fig. S5, Supplementary data). The strain produced indole-3-acetic acid (IAA, 5.85 μg/mL) and solubilized insoluble zinc compounds, including ZnO and $ZnCO_3$ (SI = 1.38 and 1.25, respectively), but did not solubilize $Zn_3(PO_4)_2$ or $Ca_3(PO_4)_2$. Siderophore production was not detected, whereas ammonia production was observed, suggesting potential plant growth–promoting effects.

Under greenhouse conditions, strain KT-9 significantly enhanced rice growth ($p < 0.05$) (Fig. 4). Bacterial suspension (cells) produced the greatest increase in plant height (33.77 cm, 57% over control,

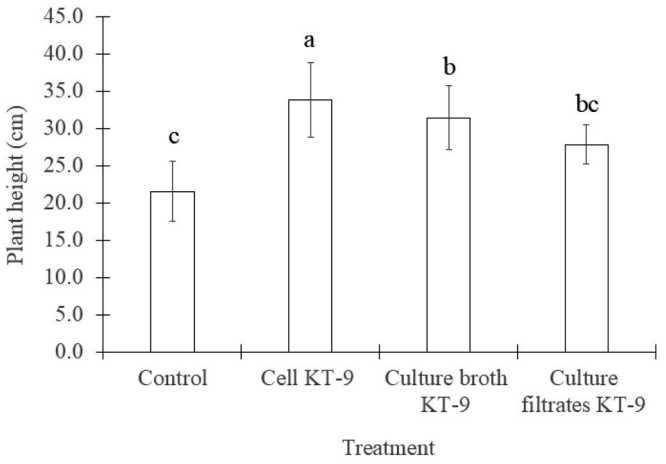

Fig. 4. Effect of bacterial suspension (1×10^7 CFU/mL), culture broth, and culture filtrates of *B. cereus* strain KT-9 on rice growth under greenhouse conditions. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey's HSD test. Data are expressed as the mean ± SD (n = 18).

21.53 cm), followed by culture broth (31.42 cm) and culture filtrates (27.82 cm). These results indicate that strain KT-9 cells and their metabolites positively influence rice growth, with the bacterial cells showing the strongest effect.

3.9. Evaluation of *B. cereus* strain KT-9 against rice sheath blight disease

All treatments significantly reduced rice sheath blight severity compared with the pathogen-inoculated control (60.49%) ($p < 0.05$) (Fig. 5). Strain KT-9 bacterial suspension achieved the lowest disease severity (27.16%) and the highest control efficacy (54.90%), outperforming culture broth (41.97%, 30.46% CE) and culture filtrates of strain KT-9 (46.91%, 22.09% CE) ($p < 0.05$). Although prochloraz and propiconazole showed slightly higher control efficacies (61.89% and 66.60%), their disease severity values (22.84% and 20.37%) were not significantly different ($p > 0.05$) from the strain KT-9 suspension, indicating comparable in planta disease suppression (Fig. 5A and B). Representative symptoms under different treatments are shown in Fig. 5C–I.

3.10. Mycolytic activity and antifungal mechanisms of *B. cereus* strain KT-9

The antifungal mechanisms of *B. cereus* strain KT-9 against *R. solani* involved both mycolytic activity and induction of oxidative stress (Fig. 6). Under nutrient-limited conditions, co-cultivation with fungal mycelia significantly enhanced bacterial growth, reaching 4.30 log CFU/mL compared with 3.32 log CFU/mL in the control without fungal biomass (Fig. 6A and B). These results demonstrate that strain KT-9 can utilize *R. solani* mycelial biomass as a nutrient source, indicating its mycolytic potential.

In addition, exposure of *R. solani* to strain KT-9 culture filtrates induced oxidative stress, as evidenced by elevated intracellular ROS levels (Fig. 6C), increased activities of antioxidant enzymes SOD and CAT (Fig. 6D and E), and alterations in glutathione redox status. Specifically, GSH increased from 0.37 to 4.15 μM/mg protein, while GSSG rose from 0.10 to 1.18 μM/mg protein, resulting in a slight decrease in the GSH/GSSG ratio (Fig. 6F–H). Collectively, these findings suggest that strain KT-9 suppresses *R. solani* through both degradation of fungal biomass and induction of oxidative stress, supporting its potential as an effective biocontrol agent.

4. Discussion

This study showed that *B. cereus* strain KT-9 exhibited strong antifungal activity against *R. solani* through multiple coordinated biocontrol mechanisms. Genome analysis also revealed several biosynthetic gene clusters potentially associated with antifungal metabolite production, suggesting a genetic basis for its antagonistic activity. Collectively, these findings indicate that strain KT-9 is a promising biocontrol agent for rice sheath blight management through multiple antifungal mechanisms.

Building on this mechanistic framework, the antifungal activity of strain KT-9 was further examined using both direct and indirect interaction assays. Notably, strain KT-9 completely inhibited fungal growth in dual culture, suggesting that direct interaction facilitates the accumulation of diffusible antifungal metabolites—such as lytic enzymes, lipopeptides, bacteriocins, and antibiotics—at inhibitory concentrations (Nguyen et al., 2025). This observation is consistent with previous studies demonstrating that diffusible and volatile compounds contribute differently to antifungal activity in microbial interactions (Guevara-Avendaño et al., 2020; Ge et al., 2025). In contrast, the reduced inhibition observed in VOC assays is likely attributable to the lower effective concentrations of VOC over distance. Nevertheless, VOC still contribute to contact-independent antifungal activity (Giorgio et al., 2015). Collectively, these findings indicate that dual culture assays reveal the maximal antagonistic potential of rhizosphere bacteria, whereas VOC assays provide complementary insights into contact-independent mechanisms underlying biocontrol.

Consistent with the role of diffusible metabolites inferred from dual culture assays, the strong, concentration-dependent inhibition exerted by strain KT-9 culture filtrates further highlights the central role of secreted bioactive compounds in fungal suppression. Near-complete inhibition at higher culture filtrates concentrations suggests the accumulation of antifungal metabolites capable of disrupting fungal growth and development. Bacterial culture filtrates has been shown to contain diffusible compounds, including secondary metabolites and cell wall–degrading enzymes, released into the surrounding environment (Abdelmoteleb et al., 2023; Ali et al., 2024; Lan et al., 2024; Santos et al., 2024). Enzymes such as chitinases and β-1,3-glucanases directly degrade fungal cell walls and compromise hyphal integrity (Prapagdee et al., 2008; Ali et al., 2022; Moremi et al., 2025). In addition to cell wall degradation, antifungal metabolites produced by *Bacillus* spp., particularly lipopeptides and peptide-based antibiotics, are known to disrupt fungal plasma membrane integrity by interacting with membrane sterols and phospholipids, leading to increased membrane permeability, leakage of intracellular contents, and impairment of essential cellular processes (Ongena and Jacques, 2008; Zhao et al., 2022). Such membrane destabilization may further stimulate intracellular ROS accumulation and oxidative damage, ultimately contributing to fungal cell death. The elevated ROS levels and altered antioxidant responses observed in *R. solani* in the present study support the involvement of oxidative stress–mediated antifungal mechanisms induced by strain KT-9 metabolites.

The broad-spectrum antifungal activity observed here further suggests that strain KT-9 produces multiple bioactive metabolites with distinct cellular targets, a characteristic commonly associated with effective biocontrol agents (Li et al., 2021b; Laisram et al., 2023). Such diversity likely reflects the presence of compounds with different modes of action, enabling suppression of a wide range of phytopathogens, as reported for other bacterial biocontrol agents, including *B. velezensis* and *Pseudomonas fluorescens*. Compared with previously described microbial biocontrol agents against rice sheath blight, strain KT-9 exhibited strong antifungal activity both *in vitro* and under greenhouse conditions. The mycelial growth inhibition caused by strain KT-9 (95.53%) was higher than that of *Lactobacillus* sp. (81.8%), *Weissella* sp. (60%), *B. velezensis* B13 (77.33%), endophytic *Bacillus* isolates (up to 79%), and *Trichoderma yunnanense* TM10 (75.43%), and was comparable to *B. velezensis* SNZC-48 (98.86%) and *Serratia marcescens* Sm85 (97.7%) (Akhtar et al.,

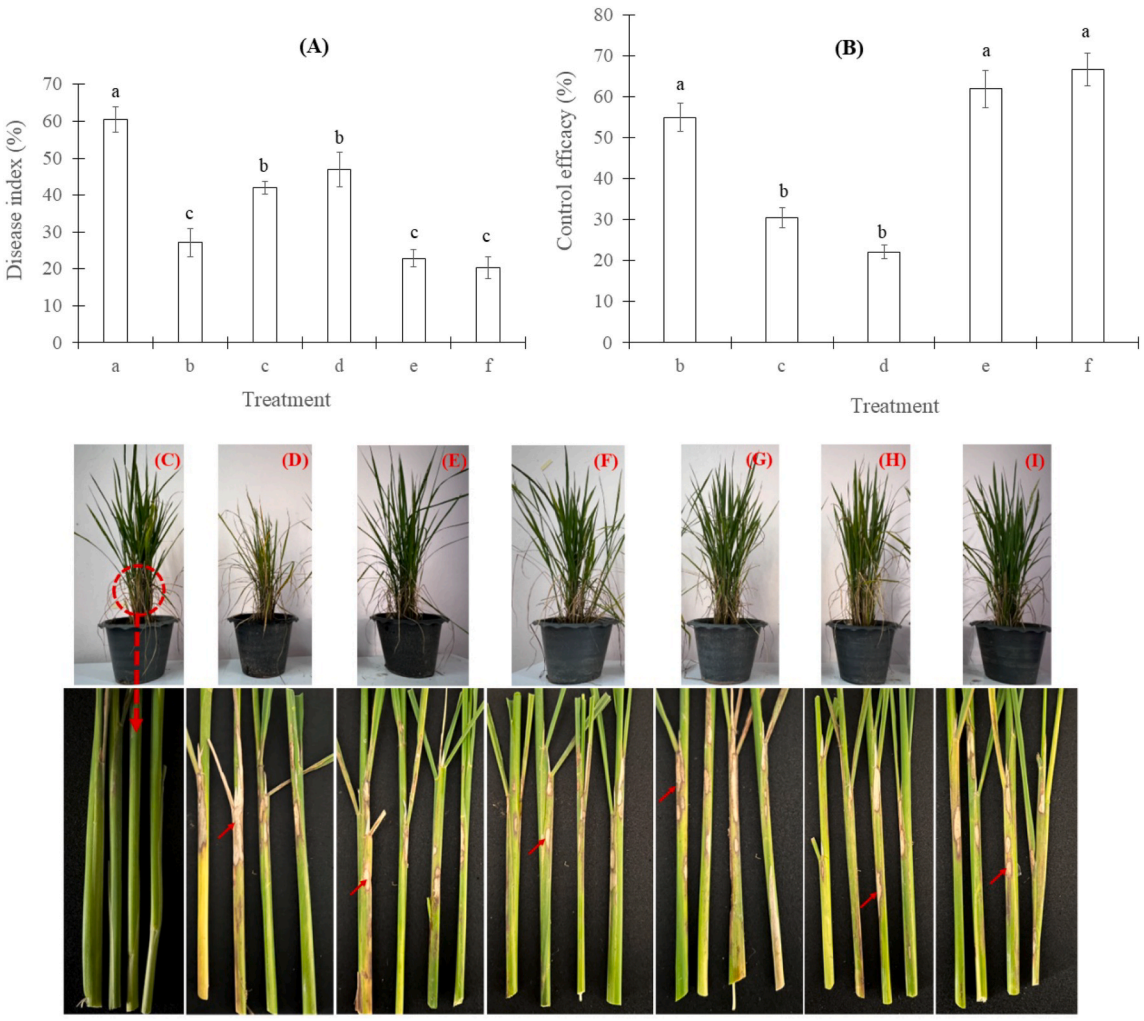

Fig. 5. Biocontrol efficacy of *B. cereus* strain KT-9 against rice sheath blight caused by *R. solani* under greenhouse conditions. **(A)** Disease index (%) and **(B)** biocontrol efficacy (%) of different treatments. Treatment codes in graphs: a = sterile distilled water (positive control); b = bacterial suspension of strain KT-9 (1×10^7 CFU/mL); c = strain KT-9 culture broth; d = culture filtrates of strain KT-9; e = prochloraz (1.0%, v/v); and f = propiconazole (1.0%, v/v). Means followed by different letters are significantly different ($p < 0.05$) according to Tukey's HSD test. Data are expressed as the mean ± SD (n = 18). **(C–I)** Representative symptoms of rice sheath blight on rice plants under different treatments: **(C)** uninoculated and untreated (negative control); **(D)** sterile distilled water (positive control); **(E)** bacterial suspension of strain KT-9 (1×10^7 CFU/mL); **(F)** strain KT-9 culture broth; **(G)** culture filtrates of strain KT-9; **(H)** prochloraz (1.0%, v/v); and **(I)** propiconazole (1.0%, v/v).

2023; Sunera et al., 2024; Jiang et al., 2025a; Naveena et al., 2025; Prismantoro et al., 2026; Zhang et al., 2026). Under greenhouse conditions, strain KT-9 reduced sheath blight severity by 54.90%, which was comparable to the disease suppression achieved by *T. yunnanense* TM10 (54.8%) and *B. velezensis* Y6 (58.67%), although lower than that reported for *B. amyloliquefaciens* (78.8%), *B. velezensis* SNZC-48 (72.38%), *P. koreensis* A1 (65.54%), and *S. marcescens* Sm85 (67.4%) (Sunera et al., 2024; Tao et al., 2024; Jiang et al., 2025a, 2025b; Prismantoro et al., 2026; Zhang et al., 2026). Nevertheless, the strong *in vitro* inhibition combined with multiple antifungal mechanisms, oxidative stress induction, mycolytic activity, and plant growth–promoting traits highlights the multifunctional biocontrol potential of strain KT-9. Notably, the antifungal efficacy of strain KT-9 culture filtrates approached that of synthetic fungicides such as prochloraz and exceeded that of azoxystrobin, although propiconazole remained more effective (Faria-Ramos et al., 2014; Rosam et al., 2020). However, unlike synthetic fungicides, strain KT-9 also exhibited plant growth–promoting activity and multifunctional biocontrol traits, which may provide additional ecological and agricultural benefits for sustainable disease management. The retention of antifungal activity after autoclaving further indicates that the active compounds are heat-stable secondary metabolites rather than thermolabile proteins (Leelasuphakul et al., 2006).

Building on the evidence that strain KT-9 culture filtrates contains heat-stable bioactive compounds with potent antifungal activity, genome mining and LC–QTOF–MS analyses were further employed to elucidate the chemical basis underlying these effects. The presence of multiple biosynthetic gene clusters, including NRPS, RiPPs, and siderophore-related clusters, indicates a strong genetic capacity for secondary metabolite production. LC–QTOF–MS analysis revealed diverse putatively identified metabolites, including cyclic peptides, diketopiperazines (DKPs), peptide-derived metabolites, aromatic acid derivatives, and maculosin (de Carvalho and Abraham, 2012; Cimmino et al., 2014; Puopolo et al., 2014; Xu et al., 2025). Compounds such as hydroxyphenyllactic acid, trans-cinnamic acid, benzoic acid, indolelactic acid, and cyclic peptide-related metabolites have previously been associated with antimicrobial or antifungal activities (Schwenninger et al., 2008; Quattrini et al., 2018; Ponzio et al., 2024).

Importantly, strain KT-9 also exhibited mycolytic behavior, degrading fungal hyphae as a nutrient source (Leveau and Preston, 2008; Nazir et al., 2010), and induced oxidative stress in *R. solani*, as evidenced by elevated reactive oxygen species (ROS) levels, altered

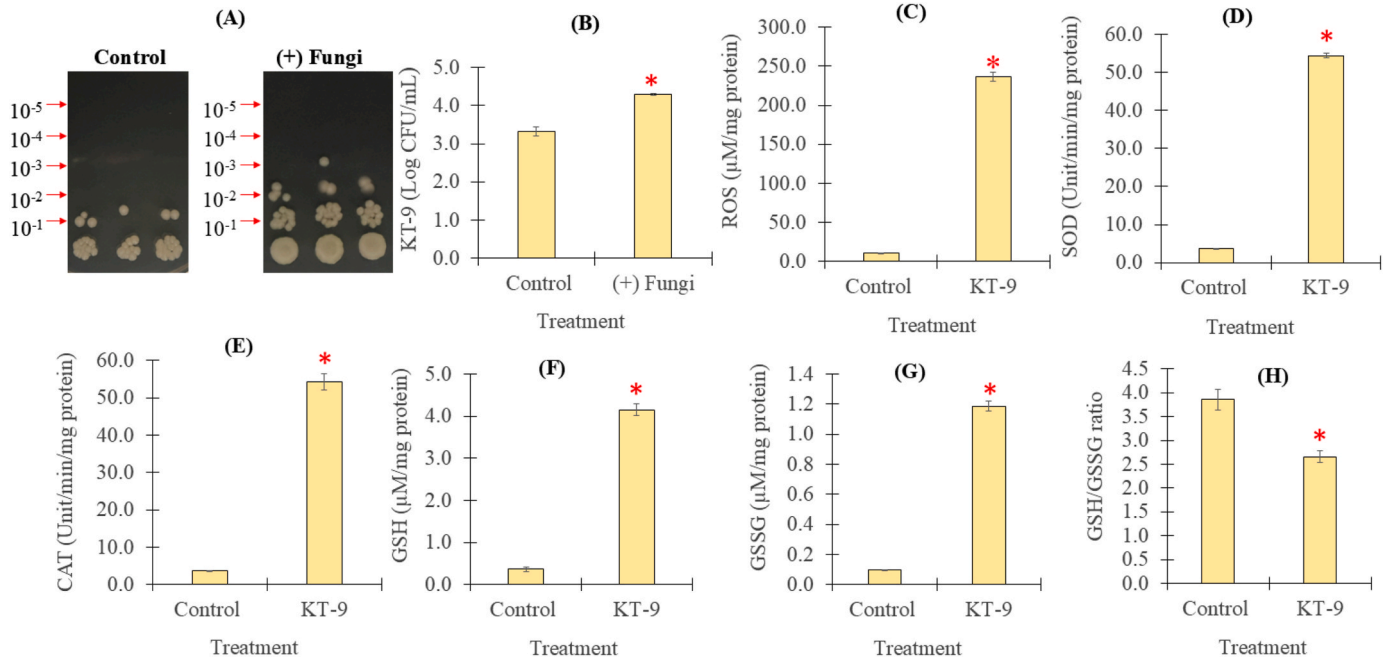

Fig. 6. *In vitro* assessment of the mycolytic activity and interaction mechanisms between *B. cereus* strain KT-9 and *R. solani*. Panels **(A)** and **(B)** show the mycolytic activity, whereas panels **(C)–(H)** present intracellular reactive oxygen species (ROS) levels, superoxide dismutase (SOD) activity, catalase (CAT) activity, reduced glutathione (GSH) content, oxidized glutathione (GSSG) content, and the GSH/GSSG ratio, respectively. Asterisks indicate statistically significant differences between treated and untreated control groups ($*p < 0.05$). Error bars represent the standard deviation (SD) of biological replicates (n = 3). Data are expressed as mean ± SD.

activities of antioxidant enzymes (CAT and SOD), and disruption of glutathione homeostasis (GSH/GSSG) (Zhao et al., 2022; Fan et al., 2023). These findings collectively suggest a coordinated antifungal strategy involving metabolite-mediated inhibition and mycolysis, with oxidative stress further contributing to fungal growth suppression.

Beyond its multi-mechanistic antifungal activity, strain KT-9 also exhibited multiple plant growth–promoting traits, further highlighting its potential as a multifunctional biocontrol agent. These included IAA production associated with enhanced root development (Khoso et al., 2024; Linda et al., 2024), zinc solubilization improving nutrient availability (Sethi et al., 2025; Upadhayay et al., 2025), and ammonia production contributing to pathogen suppression (de Andrade et al., 2023; Gupta et al., 2026). These functional traits translated into enhanced rice growth under greenhouse conditions, with bacterial suspensions showing stronger effects than culture filtrates, likely due to active colonization and continuous metabolite production. Strain KT-9 also significantly reduced sheath blight severity, demonstrating its potential as a multifunctional biocontrol agent. These findings suggest that, under greenhouse and field conditions, effective rhizosphere colonization by strain KT-9 may enhance its persistence and competitiveness in the plant-associated microbial community, enabling continuous production of antifungal metabolites in proximity to the pathogen. In addition to direct antagonism, strain KT-9 may interact with fungal pathogens through nutrient and niche competition, mycolytic exploitation of fungal biomass, and stimulation of plant defense responses. These multifaceted interactions could contribute to long-term suppression of *R. solani* and improved plant health under natural environmental conditions.

Taken together, these findings suggest that *B. cereus* strain KT-9 suppresses *R. solani* through an integrated mechanism involving (i) production of heat-stable antifungal metabolites, (ii) induction of oxidative stress and disruption of fungal redox homeostasis, (iii) ecological competition and rhizosphere colonization, and (iv) direct exploitation of fungal biomass through mycolytic activity.

5. Conclusion

B. cereus strain KT-9 demonstrated promising potential for the sustainable management of rice sheath blight caused by *R. solani*. The strain effectively reduced disease severity under semi-field conditions and exhibited plant growth–promoting traits that may enhance rice health and productivity. Overall, these findings support the potential application of strain KT-9 as an environmentally friendly biocontrol agent for rice disease management.

Funding

This research was financially supported by Songkhla Rajabhat University (Grant No. 003/2569), the Fundamental Fund (FF; Grant No. 001/2568), and the National Research Council of Thailand (NRCT; Grant No. N42A680491).

CRediT authorship contribution statement

Sawai Boukaew: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Wanida Petlamul:** Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Chetsada Kaewdee:** Investigation. **Jessdakorn Choomanee:** Investigation. **Siriporn Yossan:** Data curation, Formal analysis, Writing – review & editing. **Benjamas Cheirsilp:** Funding acquisition, Writing – review & editing. **Kanokphorn Sangkharak:** Data curation, Formal analysis, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflicts of interest, financial or personal, that could have inappropriately influenced the work reported in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2026.101382.

Data availability

Data will be made available on request.

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