Research Article

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Investigation of the mechanisms involved in the biocontrol activities of natural products from a marine soil bacterium against rice blast

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Abstract

BACKGROUND: Rice blast, caused by *Pyricularia oryzae*, is a devastating fungal disease threatening global rice production. Overreliance on chemical fungicides has raised environmental concerns and led to resistant strains, necessitating the development of sustainable alternatives. This study integrated marine microbiology and natural antifungal compounds to create eco-friendly alternatives to chemical fungicides for disease management.

RESULTS: We identified *Pseudomonas aeruginosa* R64 with broad-spectrum antimicrobial activity from mangrove soil in the Mai Po Nature Reserve. The R64 fermentation extract (RFE) exhibited multifaceted inhibition of *P. oryzae*, suppressing mycelial growth, conidiation, conidial germination and appressorial formation, while disturbing cell wall and membrane function. It also attenuated virulence by impairing appressorial penetration and invasive growth. Further chemical analysis identified phenazines and quinolines as the primary compounds in RFE, corroborated by PCR detection of corresponding phenazine biosynthetic gene clusters. Comparative bioassays with two main bioactive components of RFE, phenazine-1-carboxamide (PCN) and phenazine-1-carboxylic acid (PCA), against *P. oryzae* implicated PCN as the principal antifungal effector. RFE and PCN had higher efficacy than tricyclazole in *P. oryzae* growth inhibition, but were less effective than isoprothiolane. Furthermore, RFE and PCN displayed lower acute ecotoxicity to an environmental indicator organism than isoprothiolane, suggesting their potential as sustainable biopesticides for rice blast management.

CONCLUSION: Natural products from mangrove soil bacterium *P. aeruginosa* R64 inhibited key developmental and infection processes of *P. oryzae*, effectively reducing rice blast development. The promising disease inhibition and low ecotoxicity of mangrove-associated bacteria highlight their untapped potential for innovative, eco-friendly fungicide mining for sustainable agriculture.

Supporting information may be found in the online version of this article.

Keywords: mangrove soil bacteria; *Pseudomonas aeruginosa*; rice blast; sustainable disease management; phenazine-1-carboxamide; environmental toxicity

1 INTRODUCTION

Rice (*Oryza sativa* L.) is a staple crop that feeds over half of the world's population and its production needs to increase by ≥38% by 2030 in order to feed the growing population. However, rice production is constantly threatened by numerous diseases. Climate change and changing drought patterens caused by global warming, amd elevated carbon dioxide are further increasing outbreak risks by altering host–pathogen interactions, affecting pathogen evolution and facilitating the emergence of new pathogenic isolates.[1,2] Among these, rice blast, caused by *Pyricularia oryzae*, is one of the most devastating, responsible for 10–30% of global rice yield losses.[3–5] Current management strategies rely on resistant rice varieties and chemical fungicides. However, the prolonged use of fungicides has led to the emergence of fungicide-resistant *P. oryzae* strains, and raised concerns about

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environmental contamination, human health risks, and disruption of beneficial microbial communities in plants and rhizosphere soils.[6–11] Furthermore, breeding new resistant varieties is time-consuming, and resistance is often short-lived (typically 3–5 years) owing to genetic diversity and rapid evolution of *P. oryzae*.[12,13] Consequently, biological control has emerged as an attractive component of integrated rice blast management. Several studies have demonstrated that microorganisms such as *Bacillus* spp., *Pseudomonas* spp. and *Streptomyces* spp. exhibit antifungal activity against *P. oryzae*, both *in vitro* and *in vivo*, suggesting their potential as biopesticides.[14–21]

Most microbial natural products have traditionally been isolated from terrestrial microorganisms, yet there has been a growing recognition of mangrove originated microbes as a source of novel bioactive compound discovery in recent years.[22–24] Mangrove soil, characterized by high humidity and osmotic stress, fluctuating salinity levels, nutrient-rich sediments, as well as muddy and fine-textured soil, are renowned for their microbial and chemodiversities.[25,26] The microbial communities present in these changing environments contribute significantly to nutrient cycling, plant health and ecological balance maintenance. More importantly, they have evolved distinct metabolic pathways to produce various bioactive compounds, including those with antimicrobial activities. Many isolates of mangrove-associated species such as those of *Bacillus*, *Pseudomonas*, *Streptomyces*, *Nocardiopsis* and *Actinomycetes*, have been reported to control pathogens that may be important for human, animal and environmental health.[27,28] However, studies investigating these natural products in agricultural applications are rare, and only a few studies on suppressing rice blast and oil tea anthracnose have been reported.[24,29] Interestingly, natural product extracts from these habitats have been shown to have lower ecotoxicity to environmental indicator organisms than commercial fungicides, underscoring the potential of mangrove associated microorganisms and their secondary metabolites as an eco-friendly approach to drug discovery and biopesticides development in plant disease management.[29]

Pseudomonas spp. is a renowned group of bacteria hosting many isolates that are beneficial to plant health.[30] In particular, certain strains of the *Pseudomonas* spp. have been well-studied for their significant inhibitory effects against rice blast through various mechanisms.[14–16,31–33] One of them is to produce antifungal compounds, such as phenazines,[34–37] cyclic lipopeptide[16,38–40] and quinolines.[41] Amongst these, phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN) have been shown to have broad antifungal bioactivities against phytopathogens. Previous research has shown that PCN secreted by *P. piscium* ZJU60 suppressed the mycelia growth, mycotoxin biosynthesis, and virulence of *Fusarium graminearum* by targeting the FgGcn5 protein, a histone acetyltransferase of the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex.[42] Another study indicates that PCN can change mycelial morphology and ultrastructure of *Rhizoctonia solani* and inhibit the activities of chitin synthetase and complex I of the mitochondria electron transport chain.[43] Besides, PCA and PCN can also inhibit the growth of other phytopathogenic fungi such as *P. oryzae*,[44,45] *Botrytis cinerea*,[46,47] *Pestalotiopsis kenyana*[48] and *Colletotrichum orbiculare*.[49] However, whether PCA and PCN affect other biological processes of *P. oryzae* beyond mycelial growth inhibition, such as cell morphology, invasive structure development and full virulence, as well as their environmental compatibility, remains unclear.

In this study, we initially isolated and identified a *Pseudomonas* isolate R64 from mangrove soil in Mai Po Nature Reserve, Hong Kong, China, and found it have broad spectrum antimicrobial activities, including strong suppression on *P. oryzae* growth. This study aimed to further investigate the potential and efficacy of the natural products from R64 in rice blast suppression through step-wise characterization of its antifungal effects on *P. oryzae* mycelial growth, cell morphology, as well as host invasion and the virulence associated behaviors. Additionally, main bioactive components of the R64 natural product extract were identified and characterized by high-performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LC–MS) and biosynthetic gene cluster identification. The antifungal efficacy of R64 natural products and its main bioactive component were compared along with two commonly used fungicides with different modes-of-action (MoAs). Lastly, the ecotoxicity of the natural products from R64 and one of its main bioactive components, PCN, was assessed using an environmental indicator organism and compared with a fungicide for rice blast control. Overall, this study suggested that natural products from R64 has the potential to be developed into environmentally compatible biopesticide in rice blast management, and highlighted the importance of mangrove originated bacteria as novel biopesticide mining in the future.

2 MATERIALS AND METHODS

2.1 Reagents, strains and culture conditions

PCN (CAS: 550-89-0), PCA (CAS: 2538-68-3), commercial fungicides [tricyclazole (CAS: 41814-78-2), isoprothiolane (CAS: 50512-35-1)] and other chemical reagents (not specifically annotated) were all purchased from Shanghai Aladdin Biochemical Technology Corporation, Shanghai, China. The antimicrobial strain R64 was isolated from the mangrove soil in the Mai Po Nature Reserve, Hong Kong (114.05° E, 22.49° N), and cultured on Marine Agar 2216 medium (MA, BD Difco™). API-ZYM was used to identify the enzymatic activities of strain R64. The 16S rRNA gene of strain R64 was amplified by two universal primers (27F/1492R).[50] The gene sequence was compared to the sequences available in the NCBI nucleotide database for molecular identification. A phylogenetic tree was constructed by using MEGA 11 by the neighbor-joining method (1000 bootstrap replicates).[51] The rice bacterial leaf blight pathogen, *Xanthomonas oryzae* PXO99, and two fungal pathogens, *C. fructicola* cf1 (strawberry anthracnose) and rice blast wild-type (WT) strain, *P. oryzae* P131, were kindly provided by Dr Youliang Peng (China Agricultural University, Beijing, China). *X. oryzae* PXO99 was cultured on NA medium (5.0 g Peptone, 3.0 g beef extract, 18.0 g Agar, dissolved in double-distilled water up to 1 L, pH 7.0), whereas *C. fructicola* cf1 and *P. oryzae* P131 were cultured on OTA medium (35 g oat meal, 150 mL tomato juice, 0.7 g $CaCO_3$, 18 g agar, dissolved in double-distilled water up to 1 L, pH 7.0). All strains were maintained in an illuminated incubator (Shanghai bluepard instruments Co., Ltd, Shanghai, China) with a 12 h:12 h, light:dark photoperiod and at 28 °C.

2.2 Determination of the antagonistic activity of R64 *in vitro*

For antifungal activity tests, mycelial disks (3 mm diameter), collected from the edge of 5 ~ 7-day-old colonies, were placed on one side of a PDA plate. A 10-μL droplet of R64 suspension [optical density at 600 nm (OD_{600}) = 1.0] was inoculated onto a round

filter paper (~6 mm diameter), and placed to the opposite side of the mycelial disk. SGTYP medium without bacterial cells (5 g starch, 5 g glucose, 1 g peptone, 1 g tryptone, 1 g yeast extract and 17 g artificial sea salt dissolved in 1 L double-distilled water, pH 7.4–7.6) was used as the negative control. The plates were incubated at 28 °C and the inhibition zones were photographed at 5–7 days postinoculation (dpi) (5 days for R64 *versus C. fructicola* cf1, 7 days for R64 *versus P. oryzae* P131).

For antibacterial experiment, *X. oryzae* PXO99 suspension (OD_{600} = 1.0) was spread onto NA medium, A 10-μL droplet of R64 suspension (OD_{600} = 1.0) was inoculated onto a round filter paper (~6 mm diameter) and placed onto NA medium, SGTYP medium only without bacterial cells was used as the negative control. A 10-μL droplet of rifampicin (Rif) suspension (8 μg mL^{-1}) was used as the positive control. The plates were incubated at 28 °C and the inhibition zones were photographed at 24 h postinoculation (hpi).

2.3 Fermentation, extraction and identification of the secondary metabolite in R64

R64 fermentation extracts (RFE) were obtained according to a previous study.[24,29] Briefly, R64 cultured on MA medium was transferred to 250-mL Erlenmeyer flask containing 100 mL SGTYP medium, and fermented at 28 °C, 220 rpm for 4 days. Supernatant of the fermentation broth was then extracted three times with ethyl acetate (1:3 *v*/*v*). Finally, the fermentation crude extract was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA) at a concentration of 50 mg mL^{-1}, and stored at 4 °C until further use.

For HPLC analysis, the sample of fermentation crude extract was diluted to 2 mg mL^{-1} in DMSO and analyzed by HPLC instrument (Waters, Parsippany, NJ, USA) according to a previous study.[52] Furthermore, the sample of fermentation crude extract was sent to South China Sea Institute of Oceanology (Chinese Academy of Sciences, Guangzhou, China) for LC–MS analysis.

In order to investigate the genes involved in the biosynthesis of PCA or PCN in strain R64, five genes, including two core biosynthetic gene clusters (*phz1* and *pzh2*),[53] *phzH* (involved in the conversion of PCA to PCN),[54] *phzS* and *phzM* [transfer PCA into pyocyanin (PYO)],[54] were detected by PCR and gel electrophoresis according to a previous study.[55] The primers are listed in Table S1.

2.4 Determination of the inhibitory effects of RFE, phenazines and two fungicides on mycelial growth and morphology of *P. oryzae*

In order to assess the inhibitory rate of RFE, phenazines and two fungicides on pathogen mycelial growth, 3-mm-diameter mycelial plugs of P131 from a 7-day-old complete medium (CM) (6 g yeast extract, 3 g enzymatic casein hydrolysate, 3 g acidic casein hydrolysate, 10 g glucose, dissolved in 1 L distilled water, pH 7.0) plate were transferred to a series of CM plates containing varying concentrations of RFE (25, 50, 100, 150, 200 and 250 μg mL^{-1}, 0.5% DMSO as solvent control), phenazines (2.5, 5, 10, 15, 20 and 25 μg mL^{-1} for PCA or PCN treatments, 0.5% DMSO as solvent control), tricyclazole (50, 100, 150, 200, 250 and 300 μg mL^{-1} 0.6% DMSO as solvent control) and isoprothiolane (0.5, 1, 2.5, 5, 10 and 15 μg mL^{-1} 0.1% DMSO as solvent control). The colony diameters were measured after 5 dpi, and the inhibition rate of mycelial growth was calculated using the formula as follows: relative inhibition rate (%) = (average diameter of the control − average diameter of the compound treated strain)/average diameter of the control × 100%. All assays were conducted in triplicate with three independent tests. Furthermore, EC_{50} was calculated by IBM SPSS Statistics (v27) (IBM, Armonk, NY, USA).

Fungal mycelial morphology was examined by scanning electron microscopy (SEM) observation according to a previous study.[29] Briefly, mycelial plugs, collected from the above-described control and RFE treated plates, and were fixed in 2.5% glutaraldehyde (Macklin, Shanghai, China) overnight at 4 °C. Following fixation, the plugs were rinsed with phosphate buffer (0.1 M, pH 7.2), with each rinse lasting 15 min. Afterwards, samples were dehydrated through a series of ethanol gradients (30%, 50%, 70%, 80%, 90% and 100%) for 15 min each, followed by 100% for 20 min in absolute ethyl alcohol. The samples were then dried using supercritical carbon dioxide for 2 h and gold-coated by a sputter coating machine (E1010; Hitachi, Tokyo, Japan) for 5 min. Finally, each sample was imaged at ×5000 magnification with a high-resolution scanning electron microscope (APREO S; Thermo Fisher Scientific, Waltham, MA, USA) operating at an accelerating voltage of 5 kV.

2.5 Examination of fungal cell wall and cell membrane integrity

In order to evaluate whether RFE disturbs fungal responses to environmental stresses, mycelial plugs of P131 were transferred to CM plates supplemented with cell wall inhibitor 0.2 mg mL^{-1} Congo-red (CR; Sigma-Aldrich), 0.025% Sodium Dodecyl Sulfate (SDS), osmotic stresses (0.7 M NaCl, 1 M Sorbitol), and 10 mM H_2O_2 with or without RFE at a concentration of 100 μg mL^{-1}. The colony diameters were measured after 5 days, and the inhibition rate of mycelial growth was calculated according to the formula described in Section 2.4. All assays were conducted in triplicate with three independent tests.

The cell membrane integrity of *P. oryzae* was measured by propidium iodide (PI) staining according to the previous study with a little modification.[56] Six mycelial plugs (6 mm diameter) of *P. oryzae* were placed into CM medium. After culturing for 48 h, RFE was added to achieve final concentrations of 50 and 100 μg mL^{-1}, respectively. After an additional incubation period of 6 h, the mycelia treated with RFE or 0.2% DMSO (negative control) were collected and washed three times with double distilled water. A small portion of new hyphae developed were collected and placed on a slide containing equal volumes of 20 μg mL^{-1} PI solution (Solarbio, Beijing, China). The slide was incubated in the dark for 20 min at 28 °C before being washed three times with PBS buffer solution (50 mM, pH 7.0). Hyphal staining was observed under a fluorescent microscope (BX51; Olympus Corporation, Tokyo, Japan) with an excitation wavelength of 535 nm.

2.6 *In vitro* assessment of the inhibitory effects of RFE or phenazines on conidiation, conidial germination and appressorial formation of *P. oryzae*

For conidiation assays, *P. oryzae* was grown on OTA medium plates supplemented with 100 μg mL^{-1} RFE and 0.2% DMSO, respectively, in the dark for 2 days followed by 5 days under continuous light at 28 °C. Conidia were harvested by adding sterile distilled water and scraping the colonies with an inoculating loop. The conidia were quantified using a hemocytometer under a microscope (Olympus BX51).

For conidial germination and appressorial formation assays, conidial suspensions (1×10^5 conidia mL^{-1}) containing 0.025% Tween 20 were prepared and mixed with different concentrations of RFE (50, 100 and 150 μg mL^{-1}, respectively; 0.3% DMSO served

as the control) or phenazines (25 and 50 μg mL^{-1} for PCA or PCN treatments, 0.5% DMSO served as the control). Twenty-microliter droplets of each mixture were placed onto hydrophobic cover glass (48366-067; VWR Corp., Radnor, PA, USA), and all samples were incubated at 28 °C in darkness with a relative humidity of 100% in a growth chamber. Conidial germination rates at 2, 4, 8 and 24 hpi and appressorial formation rates at 4, 8 and 24 hpi were quantified under a microscope (Olympus BX51). The percentage of conidial germination and appressorial formation were assessed by ≥100 conidia and 100 germinated conidia for each time, respectively; each experiment was repeated two times.

2.7 Virulence assays

Rice seedlings (*Oryza sativa* cv. Lijiangxintuanheigu, 21-day-old) and barley seedlings (*Hordeum vulgare* 'E9', 10-day-old) were used for the virulence tests. For droplet inoculation, conidial suspensions (1×10^5 conidia mL^{-1}) containing 0.025% Tween 20 were prepared and mixed with different concentrations of RFE (50, 100 and 200 μg mL^{-1}, respectively; 0.4% DMSO was used as a control) or phenazines (10, 25 and 50 μg mL^{-1} for PCA or PCN treatments, 0.5% DMSO served as the control). Ten microliters of droplets of each mixture were placed onto detached rice leaves (wounded) and barley leaves, respectively. All leaves were incubated under high humidity conditions at 28 °C, following an initial 24 h dark period followed by a 12 h:12 h, light: dark photoperiod for 4–6 days. Lesion areas were calculated at 5–7 dpi using ImageJ software (National Institutes of Health, Bethesda, MD, USA). All experiments were repeated at least twice.

2.8 Determination of invasive hyphae development in barley seedlings

Conidial suspensions (1×10^5 conidia mL^{-1}) containing 0.025% Tween 20 were prepared and mixed with RFE to achieve final concentrations of 50 or 100 μg mL^{-1}, respectively; 0.4% DMSO was used as a control. Ten microliters of droplets of each mixture were placed onto detached barley leaves, which were observed under a microscope (Olympus BX51) at 32 hpi. Infectious hyphae were categorized from level I to level IV according to the previous study (I, no penetration; II, with primary invasive hyphae; III, secondary invasive hyphae do not expand to the neighboring plant cells; IV, invasive hyphae expanding into neighboring plant cells).[57] All experiments were repeated twice with >100 appressoria counted.

2.9 Ecotoxicity assessment

The ecotoxicity of RFE, PCN and isoprothiolane was evaluated using *Artemia salina* (Binzhou Evergreen Aquaculture Co., Ltd, Binzhou, China) as a model organism, following the methods described by previous studies.[29,58] Shortly, 1 g *A. salina* cysts was soaked in 500 mL artificial seawater (17 g sea salt dissolved in distilled water) for 1 h in a cone-shaped container. The container was aerated at 25 °C under continuous illumination. After 48 h, hatched nauplii were collected from the illuminated side of the container using a 20-mL micropipette. Groups of 10–15 nauplii were transferred to individual wells of a 24-well plate containing 1 mL artificial seawater and varying concentrations of RFE, PCN and isoprothiolane. The brine shrimp larvae were incubated at 25 °C for 24 h and then observed under a stereoscope. Individuals exhibiting no internal or external movement within 10 s were considered dead. All assays were conducted in triplicate with three independent tests.

2.10 Statistical analyses

Three independent experiments were performed for each assay. All the statistical analysis of experimental data was performed using Prism (v8) software (GraphPad Software, San Diego, CA, USA). Statistical comparisons were performed using one-way ANOVA. Differences were considered statistically significant at $P < 0.05$; respective significant differences were expressed as ns, *, **, etc.

3 RESULTS

3.1 Characterization and identification of strain R64 from mangrove soil

Colonies of R64 was yellowish red, uniform and smooth on MA medium plates [Supporting information Fig. S1(A)]. Gram staining revealed that R64 was a Gram-negative bacterium [Fig. S1(B)]. SEM imaging showed that R64 cells were rod-shaped and possessed flagella [Fig. S1(C)]. To preliminarily determine the taxonomic status of R64, 16S rDNA sequencing revealed that R64 (accession no. PQ380975) was closely related to *P. aeruginosa* NBRC 12689 (NR_113599.1) and *P. aeruginosa* DSM 50071 (NR_117678.1) in the same clade, with 99.93% and 99.93% nucleotide identity, respectively [Fig. S1(D)]. Additionally, the taxonomic status of R64 was determined in conjunction with physiological biochemical characterization (Table S2). The results of R64 were similar to the characteristics of *P. aeruginosa* in Bergey's Manual of Systematic Bacteriology.

3.2 *P. aeruginosa* R64 exhibited broad-spectrum antimicrobial activity

In order to explore whether R64 has antimicrobial activities against plant pathogens, *X. oryzae* PXO99, *C. fructicola* cf1 and *P. oryzae* P131 were utilized as targets. According to Figure 1, R64 formed clear inhibition zones of PXO99 on NA medium, and inhibited the mycelial growth of cf1 and P131 on PDA medium. These suggested that R64 is a bacterium with broad-spectrum antagonistic activity towards different classes of plant pathogens.

3.3 RFE inhibited mycelial growth and conidiation of *P. oryzae*

Because R64 demonstrated broad-spectrum antimicrobial activities, we suspected that this bacterium produce bioactive natural products, and thus examined the antifungal effects of its natural products extracted from the fermentation broth. As shown in Figure 2(A)–(C), the RFE inhibited mycelial growth of P131 in a dose-dependent manner. When applied at 50, 100 and 200 μg mL^{-1}, RFE caused 22.33%, 47.44% and 73.49% inhibition of fungal colony growth, respectively. Furthermore, conidiation assays revealed that the RFE significantly reduced the sporulation of P131 [Fig. 2(D)], with a 35.68% reduction noted at 100 μg mL^{-1} RFE concentration compared to the control treatment ($P = 0.0033$). These findings indicated that RFE treatment significantly affected the vegetative growth of *P. oryzae*.

3.4 RFE disturbed mycelial morphology of *P. oryzae*

Under the bright field of light microscope, hyphae from RFE-treated plates tended to be shortened and more compacted compared with the control [Fig. 2(E)]. The ultrastructure of *P. oryzae* hyphae was further observed using SEM. Although mycelia from the control group appeared as long and smooth filaments, those from the RFE-treated plates appeared twisted and aberrant, with damaged craters on the cell walls [Fig. 2(E)]. These observations

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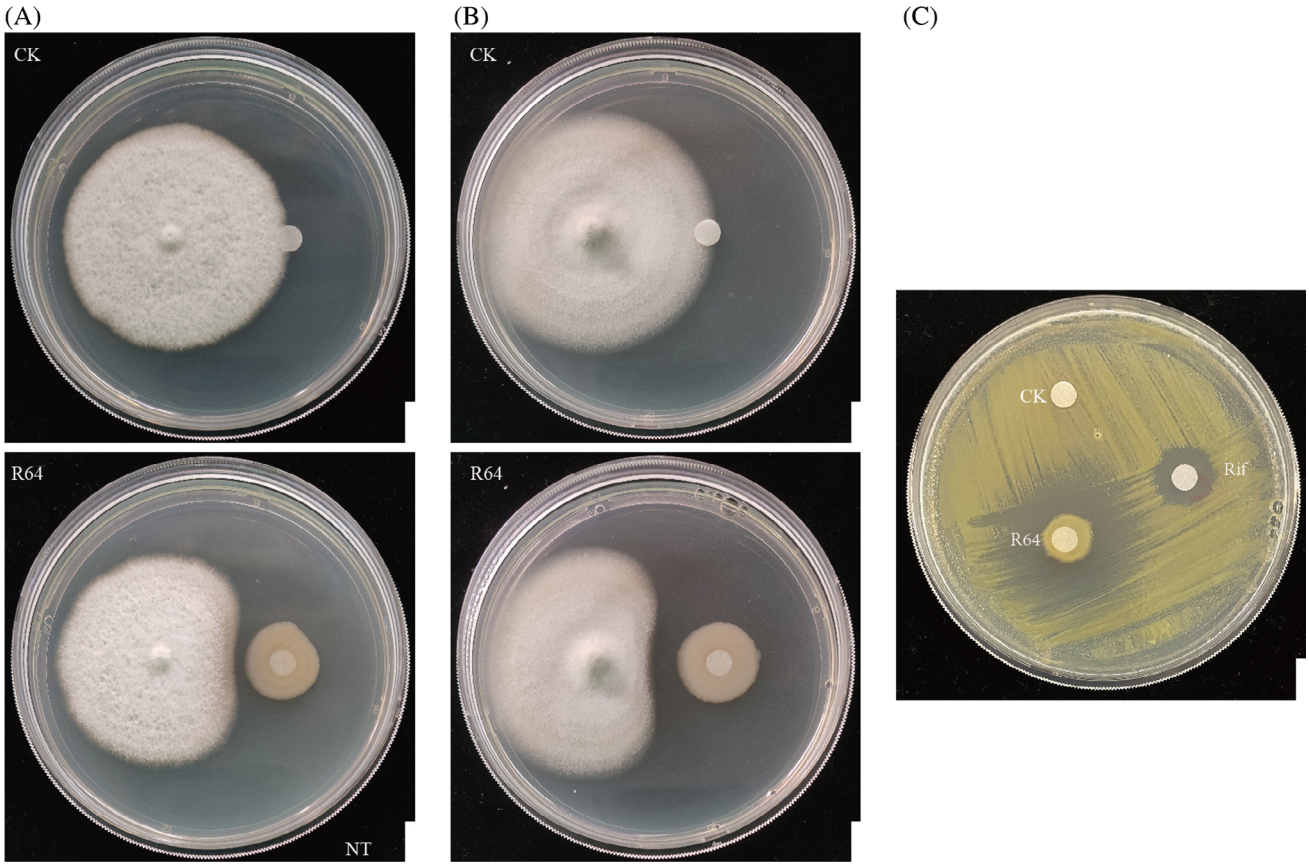

Figure 1. Antagonistic activity of strain R64 against three plant pathogens: (A) Colletotrichum *fructicola* cf1, (B) *Pyricularia oryzae* P131 and (C) *Xanthomonas oryzae* PXO99. CK, Pure SGTYP medium as control; Rif, rifampicin (8 μg mL^{-1}). Bar, 1 cm.

suggested that RFE treatment might damage the cell wall integrity and structures of *P. oryzae* hyphae, leading to abnormal morphology and growth.

The cell wall integrity (CWI) signaling pathway is important for resisting external stress and maintaining cell survival in fungi.[59,60] Next, we examined the sensitivity of *P. oryzae* to different environmental stresses to determine whether RFE disturbed cell and cell membrane wall integrity (Fig. 3). When treated with 100 μg mL^{-1} RFE, P131 became more sensitive to osmotic stresses (0.7 M NaCl, 1 M sorbitol) and oxidative stress (10 m M H_2O_2), indicating that RFE treatment might attenuate cell membrane permeability. Conversely, when exposed to cell wall inhibitors, RFE-treated strains exhibited increased resistance to 0.025% (m/v) SDS and 0.2 mg mL^{-1} CR. These results indicated that RFE treatment could interfere the cell wall integrity and change cell membrane permeability in *P. oryzae*.

In order to further verify that cell membrane function was disrupted by RFE, we used PI straining of P131 and observed by fluorescent microscopy (Olympus BX51). PI can only penetrate damaged cell membranes and stain nucleic acid in dead cells, which can be detected with red fluorescence. As expected, mycelia of the control group showed no or very faint red fluorescence, whereas RFE-treated mycelia exhibited strong red fluorescence, indicating potential damage to the cell membrane of *P. oryzae* (Fig. 4). Taken together, these results demonstrated that RFE disrupted *P. oryzae* cell wall integrity and cell membrane permeability and thereby affected its cell morphology and response to different environmental stresses *in vitro*.

3.5 RFE suppressed conidial germination and appressorial formation in *P. oryzae*

Given the above inhibitory effects of RFE on *P. oryzae*, we suspected that this natural product extract may exhibit good effects in suppressing fungal virulence, and thus examined its impacts on two preliminary steps critical for fungal infection, conidial germination and appressorial formation. Quantitative assessment results demonstrated that conidial germination reduced to 70.67%, 2.67% and 0.00% after 2 hpi treatment with RFE at concentrations of 50, 100 and 150 μg mL^{-1}, respectively [Fig. 5(A)]. Notably, at 150 μg mL^{-1} RFE, the germination rate remained below 32% even after 24 hpi. These results indicated that RFE treatment significantly inhibited the conidial germination of *P. oryzae*. Given the critical role of appressoria in *P. oryzae* host penetration,[61] the effects of RFE on appressorial formation also were examined. As illustrated in Figure 5(B), after 8 hpi, 68.37%, 7.18% and 0.00% of spores treated with RFE at concentrations of 50, 100 and 150 μg mL^{-1} (respectively) had formed appressoria, compared to 87.59% of spores treated with 0.3% DMSO forming appressoria. At 24 hpi, spores treated with 50 and 100 μg mL^{-1} RFE formed 83.93% and 55.93% appressoria, respectively, whereas the control group showed 100% appressoria formation. Notably, spores treated with 150 μg mL^{-1} RFE failed to form appressoria at 24 hpi. These findings indicated that RFE can significantly inhibit conidial germination and appressorial formation in *P. oryza*e, and even conidia could be formed, some of them could not develop into the next infectious stage.

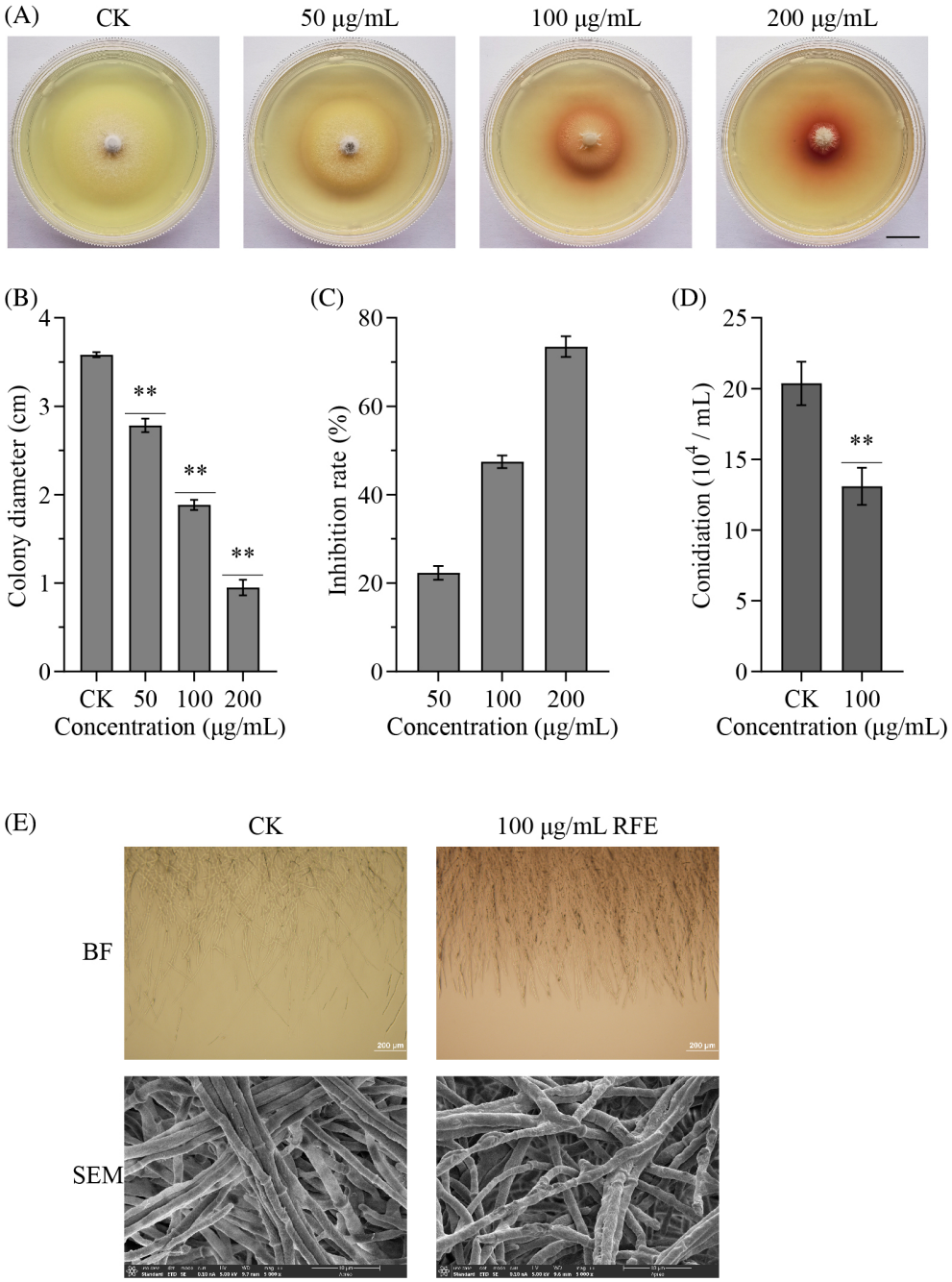

Figure 2. Effects of R64 fermentation extract (RFE) on vegetative growth, conidiation and mycelial morphology of *Pyricularia oryzae*. Colony (A) growth and (B) diameters of *P. oryzae* and (C) growth inhibition rates on CM medium treated with varying concentrations of RFE for 5 days. Bar, 1 cm. (D) Conidiation and (E) mycelial morphology of *P. oryzae* on OTA medium with or without 100 μg mL^{-1} RFE. CK, 0.4% DMSO as solvent control; BF, bright field; SEM, scanning electron microscope. All assays were conducted in triplicate with three independent tests. The statistical analyses were performed using one-way ANOVA. Double asterisks (**) represent a significant difference ($P < 0.01$).

3.6 RFE treatment impaired *P. oryzae* virulence and host penetration

Following the observed impairment of infectious structure development in *P. oryzae* by RFE treatment, we investigated its effect on the pathogen's virulence. The results of droplet inoculation demonstrated that RFE inhibited lesion formation on both rice and barley leaves in a dose-dependent manner [Fig. 6(A),(B)]. Statistical analysis revealed that RFE significantly reduced lesion size at 5 dpi in a dose-dependent manner in rice leaves [Fig. 6(C)]. Although typical blast lesions had developed in the control group with an average size of ~0.204 cm^2, the average lesion area in the 200 μg mL^{-1} RFE treatment was only ~0.038 cm^2, which was more than ~80% reduced compared to the control group ($P < 0.01$). These results demonstrated that RFE damaged the full virulence of *P. oryzae* in both rice and barley.

In order to elucidate the mechanism behind reduced pathogenicity by RFE, we conducted a barley infectious assay, which is a classic model used to observe different developmental stages of infectious hyphae in *P. oryzae*. RFE treatment significantly reduced both the formation of penetration structures on barley

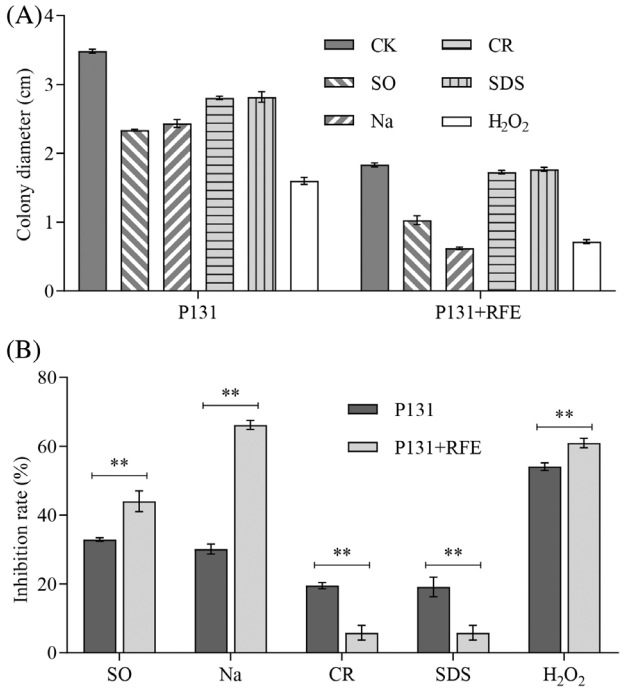

Figure 3. Effects of R64 fermentation extract (RFE) on *Pyricularia oryzae* response to different environmental stresses. (A) Colony diameters of *P. oryzae* and (B) growth inhibition rates on CM medium plates supplemented with 100 μg mL^{-1} RFE in combination with different environmental stresses. CK, 0.2% DMSO as a solvent control; HO, 10 mM H_2O_2; CR, 0.2 mg mL^{-1} Congo-red; SDS, 0.005% Sodium dodecyl sulfate; So, 1 M Sorbitol; Na, 0.7 M NaCl. All assays were conducted in triplicate with three independent tests. The statistical analyses were performed using two-way ANOVA. Double asterisks (**) represent a significant difference ($P < 0.01$).

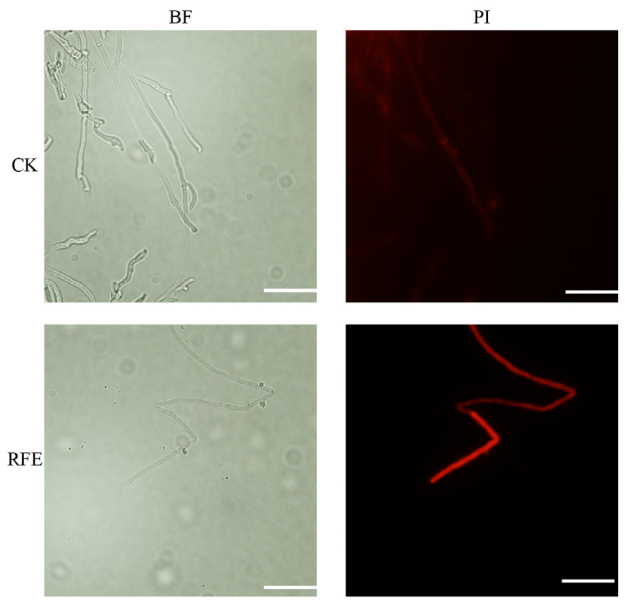

Figure 4. Propidium iodide (PI) staining of *Pyricularia oryzae* treated with 100 μg mL^{-1} RFE. CK, 0.2% DMSO as a solvent control; BF, bright field. Bar, 50 μm.

leaves as well as the expansion of infectious hyphae in the host cells. Penetration data [Fig. 6(D),(E)] showed that at 30 hpi, >85% of P131 conidia adhered to the host tissue surface had

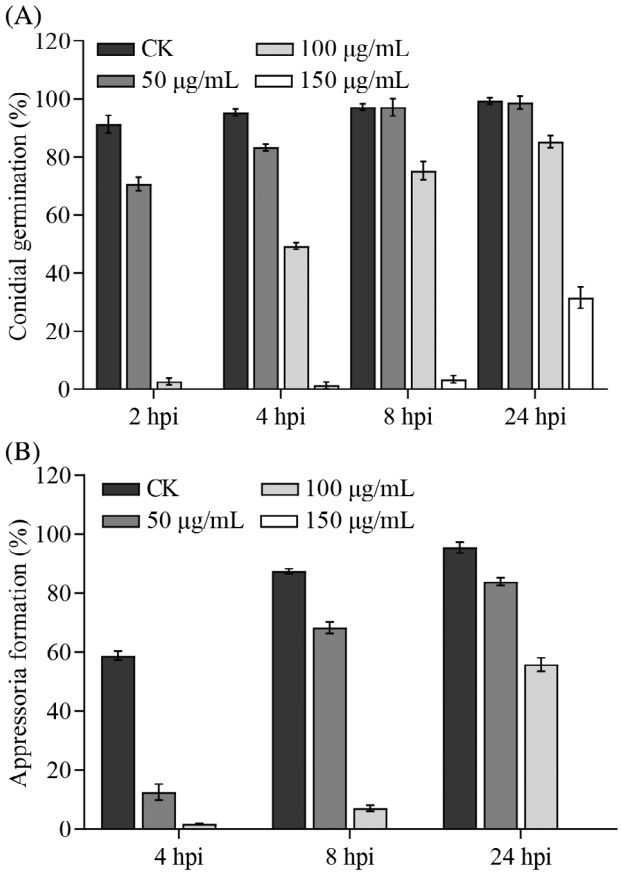

Figure 5. Inhibitory effects of R64 fermentation extract (RFE) on conidial germination and appressorial formation of *Pyricularia oryzae*. (A) Conidial germination and (B) appressorial formation rates of *P. oryzae* treated with varying concentrations of RFE on the hydrophobic surface at different time points. CK, 0.3% DMSO as solvent control. All experiments were repeated twice with >100 conidia counted.

formed infectious hyphae in the control group (Type II–IV), whereas >90% of conidia treated with RFE (100 μg mL^{-1}) formed appressoria only (Type I). Both the infection rate and invasive hyphae development of RFE-treated strains was significantly lower and delayed, compared to those of the DMSO-treated group. Therefore, RFE treatment could effectively inhibit rice blast by disrupting host penetration and subsequent invasive hyphae development of *P. oryzae*.

3.7 Phenazines as key bioactive components of RFE against *P. oryzae*: superior antifungal effects of PCN compared to PCA

In order to identify the antimicrobial substances in RFE, we performed HPLC and LC–MS analyses (Fig. S2). Results revealed six primary compounds belonging to two classes (phenazines and quinolones) (Table S3), among which the m/z values of two phenazine derivatives, including the m/z 225.0666 $[M + H]^+$ of PCA and the m/z 224.0816 $[M + H]^+$of PCN, were consistent with the previous studies.[62,63] Furthermore, PCN and PCA together took account of more than 70% of the total RFE as revealed by peak areas from the HPLC analysis [Fig. S2(A)]. Additionally, PCR analysis confirmed that strain R64 contained several genes related to phenazines biosynthesis (Fig. S3), including *phz1*, *phz2*, *phzH*, *phzS* and *phzM*,[53,55] further supporting the presence of PCN and PCA in RFE.

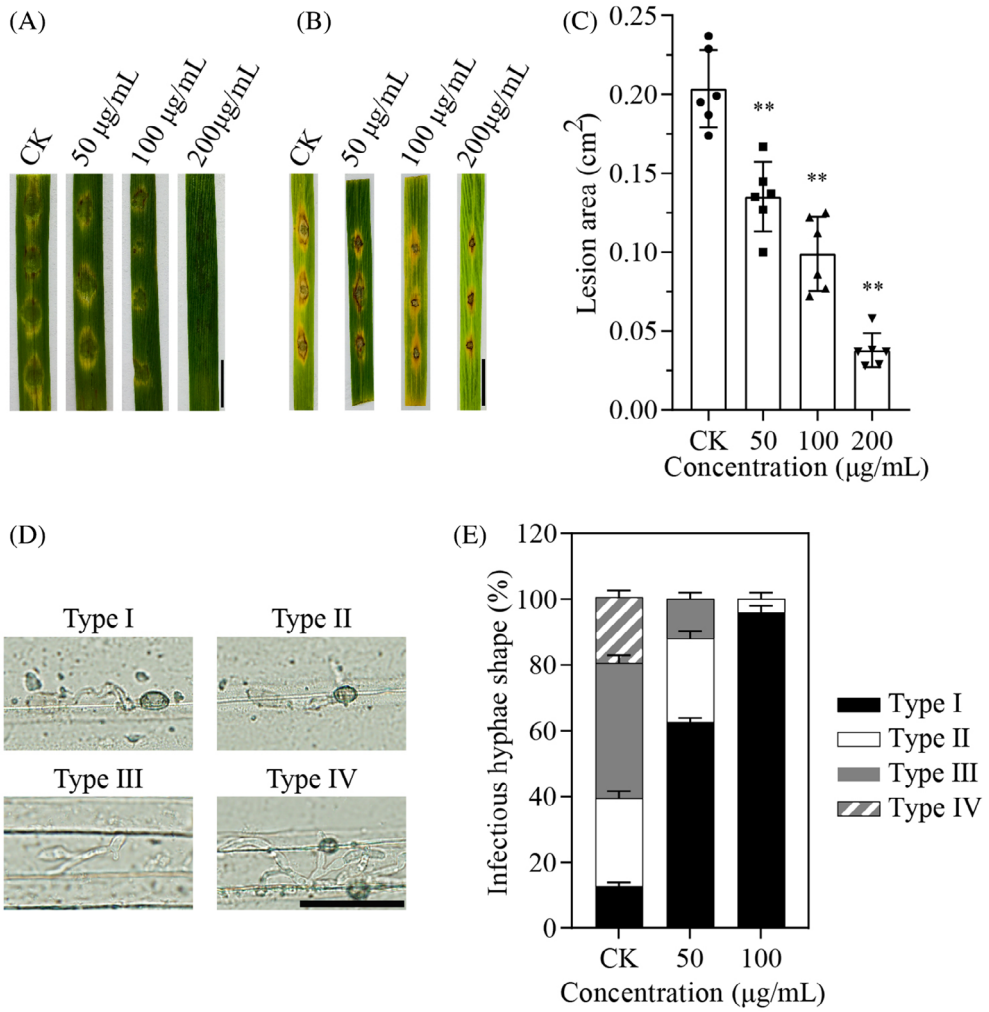

Figure 6. Inhibitory effects of R64 fermentation extract (RFE) on full virulence and invasive hyphae development of *Pyricularia oryzae*. Droplet-inoculation of *P. oryzae* on detached (A) barley and (B) wounded rice leaves. Bar, 1 cm. (C) Lesion areas in rice leaves treated with different concentrations of RFE after 5 days. Mean area were calculated from $n = 6$ biologically independent blast lesions 5 d postinfection. The experiment was repeated twice with similar results. The statistical analyses were performed using one-way ANOVA. The double asterisks (**) represent significant difference ($P < 0.01$). (D) Representative images of infectious hyphae of four different categories. I, no penetration; II, with primary invasive hyphae; III, secondary invasive hyphae do not expand to the neighboring plant cells; IV, invasive hyphae expanding into neighboring plant cells. Bar, 20 μm. (E) Quantitative analysis of invasive growth in barley leaves at 30 hpi. Mean percentage of invasive hyphal growth was calculated based on an analysis of 50 appressorium penetration sites. CK, 0.4% DMSO as a solvent control.

Previous reports have shown that PCA had antifungal activities against *P. oryzae* and PCN could induce systemic resistance against rice blast.[33,37,44,45] To determine the potential of these compounds in rice blast management, we compared their antifungal activities against *P. oryzae*. The results demonstrated that at 25 μg mL^{-1}, PCN and PCA inhibited mycelial growth of *P. oryzae* by 90.26% and 19.60%, respectively (Fig. 7). Further analysis of conidial development on hydrophobic cover glass revealed that at 50 μg mL^{-1}, average germination rates were ~72% in the PCA treatment but only ~0.67% in the PCN treatment, respectively [Fig. 8(A),(B)]. Moreover, appressorial formation rates were ~64% in the PCA group but 0% in the PCN group at 50 μg mL^{-1} [Fig. 8(C)]. Taken together, these results suggested that PCN had stronger antifungal effects against *P. oryzae*.

In accordance with these results, PCN treatment of 50 μg mL^{-1} almost completely inhibited the formation of lesions on both barley and rice leaves, which was significantly stronger that of PCA of the same concentration (Fig. 9). These results collectively suggested that phenazines, especially PCN, was the principal antifungal effector in RFE.

The antifungal efficacy of PCN and RFE also was compared with two commercial fungicides. As shown in Table S4, the half maximal effective concentration (EC_{50}) of PCN against *P. oryzae* was ~13.711 μg mL^{-1}, which was ~7.5- and 10.6-fold lower than that of RFE (102.943 μg mL^{-1}) and tricyclazole (145.517 μg mL^{-1}), respectively, yet ~five-fold more than that of isoprothiolane (2.766 μg mL^{-1}). These results indicated that PCN and RFE had good antifungal efficacy better or comparable to certain fungicides.

3.8 Reduced toxicity of RFE and PCN

Environmental toxicity assessments revealed that both RFE and PCN exhibited significantly lower toxicity to *A. salina* compared to isoprothiolane. At 100 μg mL^{-1}, a concentration that RFE showing ~50% rice blast suppressive effects in both barley and rice leaves [Fig. 6(A),(B)], this natural product extract displayed

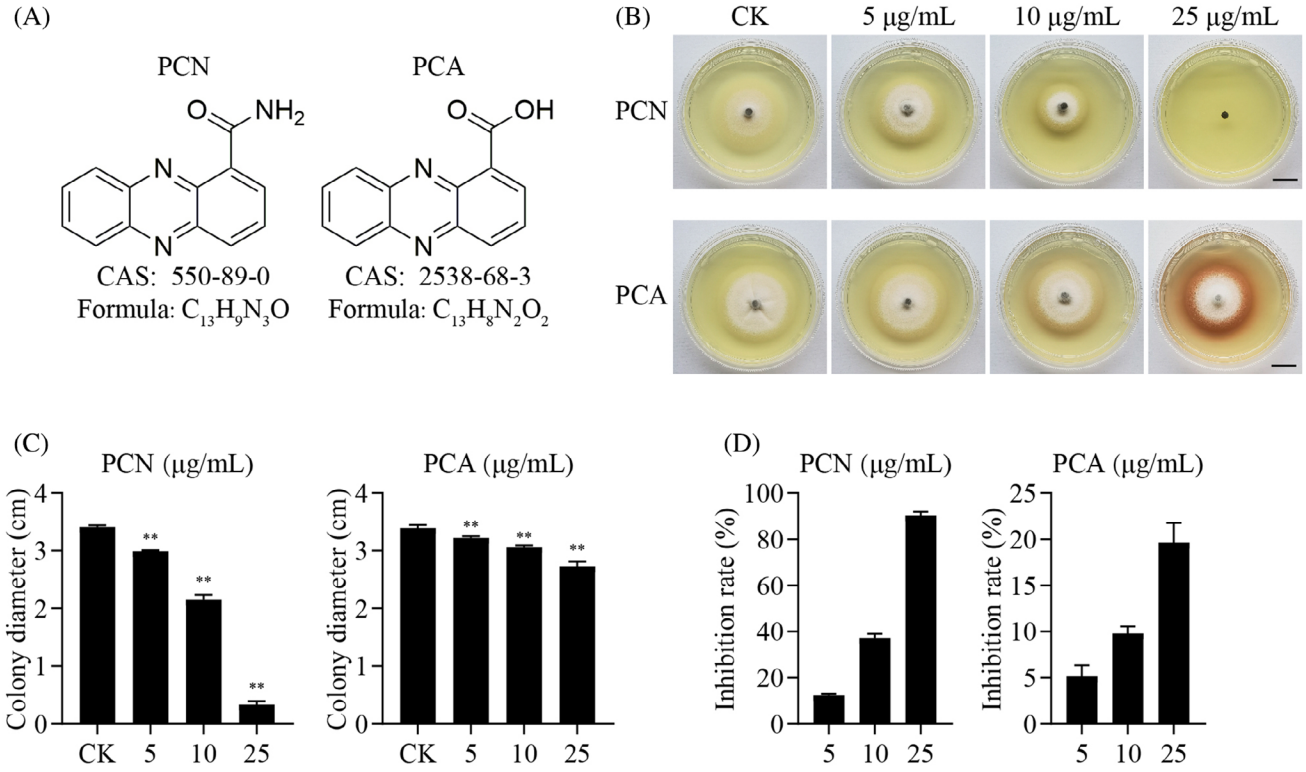

Figure 7. Effects of phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN) on mycelial growth of *Pyricularia oryzae*. (A) Chemical structure of PCA and PCN. (B) Growth and morphology of *P. oryzae* treated with PCA or PCN at different concentrations. Bar, 1 cm. (C) Colony diameters and (D) inhibition rates of PCA and PCN at varying concentrations on P131 colony growth. CK, 0.5% DMSO as solvent control. All assays were conducted in triplicate with three independent tests. The statistical analyses were performed using one-way ANOVA. Double asterisks (**) represent a significant difference ($P < 0.01$).

moderate toxicity, causing ~42.53% death of *A. salina* [Fig. 10(A)]. Likewise, PCN of 5 and 10 μg mL^{-1} caused slight toxicity to *A. salina*, resulting in ~15.78% and ~20.28% death, respectively [Fig. 10(B)]. Even at 25 μg mL^{-1}, under which PCN almost completely suppressed rice blast development, the compound only caused ~42.03% *A. salina* death. By contrast, isoprothiolane at 25 μg mL^{-1} led to >80% mortality rates [Fig. 10(C)]. Considering that 172 μM (50 μg mL^{-1}) isoprothiolane showed only 69% inhibition rate in rice blast development,[64] both RFE and PCN had significantly lower toxicity to *A. salina*.

4 DISCUSSION

Bacteria from different specific marine habitats, such as mangrove soils, coral reefs and deep-sea hydrothermal vents, are emerging as important reservoirs of biologically active compounds.[24,65,66] Because of their adaptation to extreme marine environments, many marine bacteria harbor biosynthetic pathways with high biodiversity to enable them to produce bioactive metabolites with unique structures and novel MoAs.[67–69] As such, the bioprotective potential of marine microorganisms and their derived natural products in plant disease control has started to come forward. For example, a novel antifungal compound from the marine red algal-derived fungus *Penicillium chermesinum* EN-480, chermesiterpenoid C, had been reported to have good activity against the plant pathogenic fungus *C. gloeosporioides* with a minimum inhibitory concentration (MIC) value of 16 μg mL^{-1}.[70] Plipastatin A1, produced by a marine sediment-derived *B. amyloliquefaciens* SH-B74, significantly reduced the lesion size of gray mold disease in tomato plants at the concentration of 50 μM.[71] Furthermore, a review from Li *et al.*[72] discussed the isolation, identification and antimicrobial mechanisms of bioactive natural products from marine microorganisms from different sources (such as slime, seaweed, sponge and microalgae). These active natural products showed strong inhibition on different plant pathogens. For example, roridin A and roridin D, isolated from *Myrothecium* sp. fungus isolated from sponge, exhibited great inhibition on plant fungal pathogens, such as *P. oryzae* and *Sclerotinia sclerotiorum*.[73] However, to the best of our knowledge, there has been no study exploring mangrove soil associated microorganisms as biocontrol agents in rice blast suppression.

In this work, the strain, named R64, was selected from mangrove soil in Mai Po Nature Reserve in Hong Kong. Through the morphological, biochemical identification and 16 s rRNA sequencing analysis, R64 was identified as *P. aeruginosa*, which belongs to a renowned bacterial group hosting many biocontrol agents. One potential issue of using live microorganisms in plant disease management is that the biocontrol efficacy is highly dependent on the interaction among the biocontrol agents, hosts and the pathogens, and the survival and persistence of the biocontrol agents are subject to environmental changes. However, the bioactive compounds from the producing microorganisms can be directly applied in plants, and the disease inhibition efficacy tend to be more repeatable at given concentrations and applying methods. In this study, the yield of RFE by small-scale fermentation using synthetic medium was ~250 mg L^{-1} (~25 mg L^{-1} for PCN). Although this may not be comparable to commercial fungicides or PCN at this stage, it is feasible to further increase the yield of

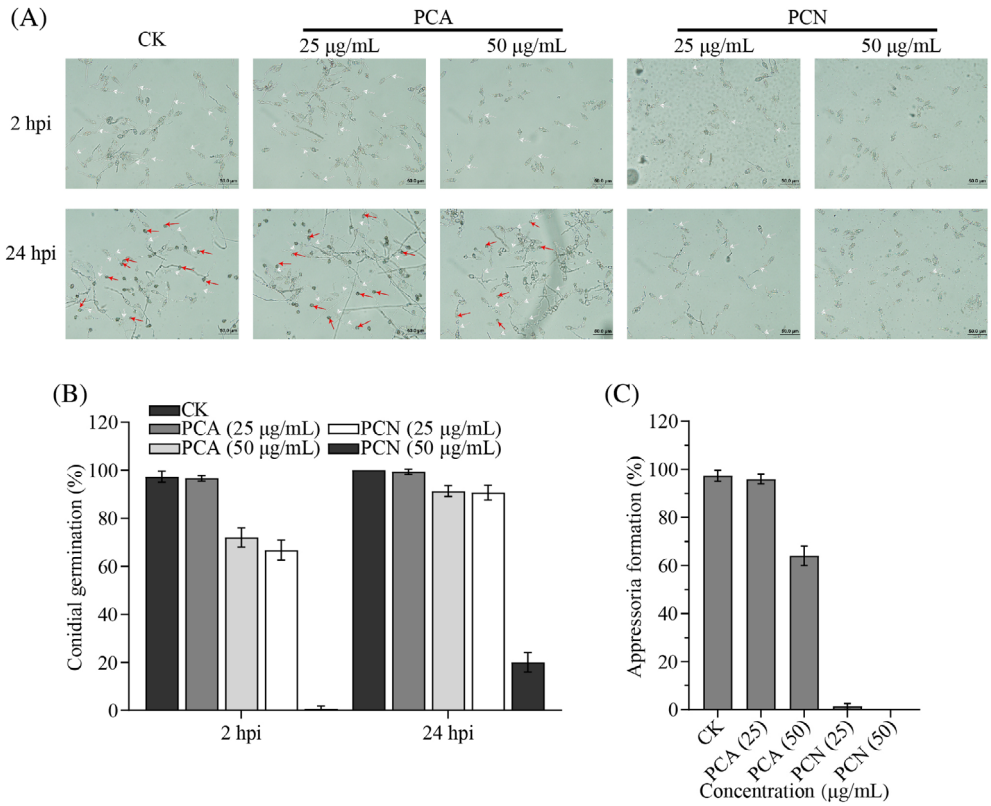

Figure 8. Inhibitory effects of phenazine-1-carboxylic acid (PCA) or phenazine-1-carboxamide (PCN) treatment on conidial germination and appressorial formation of *Pyricularia oryzae*. (A) Bright field images of *P. oryzae* conidia treated with different concentrations of PCA or PCN at 2 and 24 h postinoculation (hpi). White arrows indicate germ tubes, red arrows indicate appressoria. Bar, 50 μm. (B) Percentages of conidial germination at 2 and 24 hpi. (C) Appressorium formation rates at 24 hpi. CK: 0.5% DMSO as solvent control. All assays were conducted in triplicate with three independent tests.

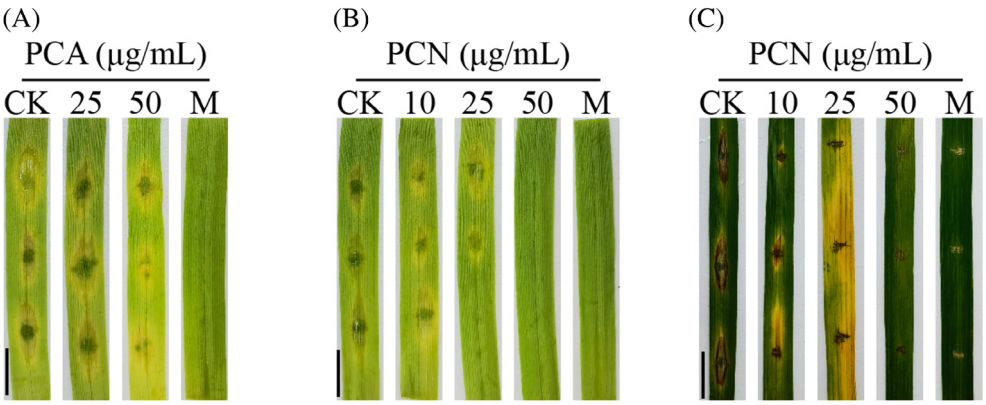

Figure 9. Inhibitory effects of phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN) on the full virulence of *Pyricularia oryzae*. Droplet-incubation of *P. oryzae* treated with (A) PCA and (B) PCN on detached barley leaves. Bar, 1 cm. (C) Droplet-incubation of *P. oryzae* treated with PCN on detached rice leaves. Bar, 1 cm. All assays were conducted in triplicate with three independent tests. The statistical analyses were performed using one-way ANOVA. Double asterisks (**) represent a significant difference ($P < 0.01$).

RFE or PCN by optimizing medium composition and other factors affecting fermentation such as time, temperature and aeriation.[74,75] Utilizing strains with desired or modified genetic background is another commonly used strategy to boost bioactive compound production. For example, the yield of PCN in *P. chlororaphis* H5 *fleQ relA* strain was more than six-fold that of the original WT strain. By utilizing this mutant and optimizing the fermentation conditions, PCN production can be enhanced by >10-fold.[76] Therefore, further investigation of the most optimal conditions and scale-up production of RFE and its bioactive components by the above-described approaches may further increase their yields and cut down the cost.

Pseudomonas aeruginosa is known to produce a number of secondary metabolites with potent antibiotic activities, such as

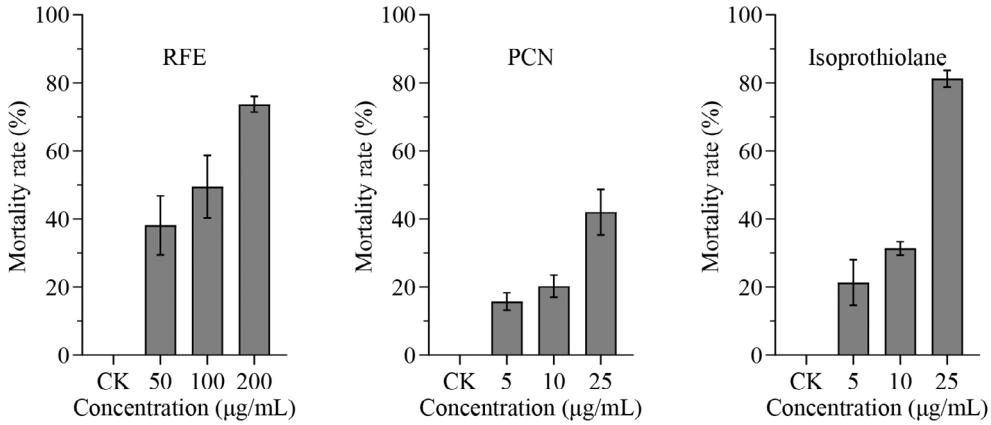

Figure 10. Ecotoxicity assessment of R64 fermentation extract (RFE), phenazine-1-carboxamide (PCN) and isoprothiolane. CK, 0.5% DMSO as solvent control. Al l assays were conducted in triplicate with three independent tests.

phenazines, rhamnolipids, pyocyanin and various siderophores.[77–79] Although known as opportunistic pathogens, bacteria of this genus display extremely high genetic variation, metabolic versatility, and pathogenicity difference. Additionally, a number of *P. aeruginosa* isolates have been reported to show broad-spectrum antimicrobial activity against plant pathogens, promote plant growth and increase plant resistance, exhibiting great potential in agricultural application.[80–82] Furthermore, it has been reported that *P. aeruginosa* isolates from agricultural soil generally display different metabolic profiling, and less virulence and antibiotic resistance compared to those of clinical origins.[83,84] Therefore, isolates of this genus should be carefully and comprehensively investigated case by case before their application. Interestingly, the confrontation test results of R64 against the rice pathogens *X. oryzae* PXO99 and *P. oryzae* P131, as well as another common ascomycete pathogen, *C. fructicola* cf1, suggested that it can produce natural compounds with broad-spectrum antimicrobial activities, which is consistent with previous reports.[49,82] Therefore, we used the crude extracts of the R64 secondary metabolites in subsequent studies to examine its potential as biopesticide in rice blast management. RFE of ≥100 μg mL^{-1} showed significant antifungal effects on *P. oryzae*, by inhibiting the vegetative growth, conidiation, conidial germination and subsequent appressorial formation in a multifaceted manner. These together led to significant disease inhibition rate of ~51.35% in rice leaves in the RFE treatment at 100 μg mL^{-1}. These results corroborated previous reports on the antifungal activity of secondary metabolites derived from other *Pseudomonas* isolates.[85,86]

Initial observation of mycelial morphology on PDA plates by compound microscope revealed that RFE-treated ones became more compacted and shortened. Further observation by SEM showed that these mycelia tended to be shortened with more nodes protruding and had obvious wrinkled and rough surface compared to the long, smooth filaments in the control group. These suggested possible alteration in cell morphology upon RFE treatment, which led us to examine the functions of fungal cell wall and membrane. The fungal cell wall is the outermost structure in maintaining cell integrity, and plays critical role in environmental resistance. Meanwhile, cell membrane is a major barrier responsible for controlling the flux of substances into and out of the cell. Disruption of these structures could drastically impair environmental resistance and nutritional acquisition in *P. oryzae*.[59,60,87] As expected, we observed increased sensitivity of *P. oryzae* to osmotic and oxidative stresses when RFE was applied, suggesting its disruption of some cellular processes vital for *P. oryzae*. Interestingly, we observed an opposite trend when *P. oryzae* was subject to cell wall inhibitors and observed increased growth of *P. oryzae* upon RFE treatment. This was possibly a consequence of the fact that RFE treatment might interfere the cell wall integrity pathway and cell membrane integrity in different ways, and similar results have been reported before.[88]

Further LC–MS analysis of the bioactive components of RFE led to the identification of six compounds, with two known compounds, PCN and PCA, being the major ones, accounting for >70% of the total RFE [Fig. S2(A)]. Further PCR analysis confirmed the presence of all known phenazine biosynthesis genes in R64, including *phz1*, *phz2*, *phzH*, *phzS* and *phzM*. These results are consistent with previous reports indicating the antifungal activity of phenazines secreted by *Pseudomonas* species.[53,55,89] For instance, various phenazines, such as PCA, PCN, 1-hydroxy-PCA, PCA and phenazine-1,6-dicarboxylic acid had been reported to exhibit *in vitro* antifungal activities against *B. cinerea*, *R. solani* and *F. culmorum*.[53] Likewise, PCN produced by *P. aeruginosa* NF011showed excellent antifungal activity against *F. graminearum*, and its treatment led to induced systemic resistance against *Fg* spores infecting wheat.[89] The comparison between PCN and PCA inhibition on fungal growth also suggested that the major bioactive component of R64-FRE was likely to be PCN, because this compound could completely suppress fungal growth at 25 μg mL^{-1}, whereas PCA showed a much weaker inhibition rate when applied at the same concentration. Similar to RFE, both PCN and PCA exhibited multifaceted inhibitory effects on *P. oryzae* development and virulence and inhibited disease development in rice leaves to different extent. Because currently there are no studies reporting the functional mechanisms of these compounds in against *P. oryzae* nor their effects on plants, further mechanistic investigation of these compounds together with RFE by approaches such as transcriptomic profiling, gene expression analysis, fungal mutant studies and plant development assessment would be useful to facilitate their future application in rice blast management.

Comparison of the antifungal efficacy of PCN, RFE with two commercial fungicides suggested that both PCN and RFE had better efficacy than tricyclazole but were less effective than isoprothiolane. We then went forward to evaluate the environmental compatibility of PCN, RFE and isoprothiolane, as this is a critical consideration in disease control strategy

development in sustainable agriculture. In our case, we conducted initial environmental toxicity assessment using a common bioindicator. *A. salina* is a widely distributed zooplanktonic crustacean frequently used in toxicity assessments owing to its sensitivity to environmental pollutants and relevance to aquatic ecosystems. Our findings demonstrated that RFE and PCA exhibit significantly lower environmental toxicity compared to isoprothiolane, a commonly used fungicide. As shown in Figure 10, RFE at 100 μg mL^{-1} could lead to ~50% disease inhibition, and PCN at 25 μg mL^{-1} could almost completely suppress disease development. Meanwhile, these two treatments only caused ~50% death of *A. salina* under the above-described concentrations. However, studies have shown that 172 μ M (~50 μg mL^{-1}) isoprothiolane can only cause 69% inhibitory efficacy in leaf blast.[64] However, 25 μg mL^{-1} isoprothiolane resulted in >80% death of *A. salina*. This aligns with previous research suggesting that natural products from mangrove-associated bacteria generally have reduced negative environmental impacts.[29]

Hereafter, it was revealed that mangrove soil-derived *P. aeruginosa* R64 and its metabolites, especially for PCN, displayed a promising prospect for being a biopesticide of rice blast disease. According to the Guidance on the risk assessment of metabolites produced by microorganisms used as plant protection active substances published by European Commission, a series of criteria and questions need to be addressed before any microbial metabolites can be used in agricultural scenarios to collect sufficient information of their impacts on both target and nontargeted organisms.[90] Therefore, whether RFE has any negative impacts on other environmental organisms and human health remains to be further investigated. In the past decade, >1000 novel natural products have been discovered and identified from mangrove sources, and this number is still increasing. However, compared to the application in the medical field, their application in agriculture remains poorly characterized. Our findings support the exploration of marine microbial bioresources as a novel, sustainable approach for agricultural pest management, which is understudied as present. Future research to elucidate the functional mechanisms involved in RFE and PCN, determine their biocontrol efficacy in field, and examine their impacts on plants and other environmental organisms as well as in-depth analysis of the structure–activity relationships of PCN to optimize its biocontrol efficacy, would contribute to the development of novel, effective and sustainable strategies to combat rice blast.

5 CONCLUSIONS

To the best of our knowledge, *P. aeruginosa* strain (R64 in this study) with broad-spectrum antimicrobial activity was isolated from mangrove soil for the first time. The RFE demonstrated significant inhibitory effects on mycelial growth, disrupted cell wall integrity and cell membrane permeability, and disturbed conidial development in *P. oryzae*. RFE also effectively blocked appressorial development and the formation of infectious hyphae of *P. oryzae*, thereby impairing the pathogen's virulence in rice leaves. Through HPLC, LC–MS analyses combining biological assays, we confirmed that phenazines, particularly Phenazine-1-carboxamide (PCN), was the main active component of RFE against *P. oryzae*. Interestingly, it is the first time to elucidate the effects of PCN on the biological processes of *P. oryzae*, such as vegetable growth and pathogenicity. Moreover, both RFE and PCN show significant lower ecotoxicity than a commercial fungicide widely used for rice blast control. Further research into the practical applications of these marine-derived compounds could contribute to the development of novel, environmentally friendly strategies for crop protection.

AUTHOR CONTRIBUTIONS

Liwang Fei: Writing–original draft and editing, visualization, validation, resources, data curation, conceptualization. Rahila Hafeez: Writing and review. Junliang Zhang: Investigation, data curation (LC–MS analysis). Shiquan Fu: Investigation, data curation. Ying Xu: Project administration, funding acquisition. Lingyun Hao: Writing–review & editing, Supervision, Project Administration, Funding Acquisition, Conceptualization. All authors reviewed and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

We declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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