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Full Length Article

Fungicidal Activity and Biological Characteristics of a Novel Natural Product Fungicide: Phenazine-1-carboxamide-derived 18-1

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Abstract

A novel phenazine-1-carboxamide-derived 18-1 (PCND 18-1) was evaluated in terms of its potential for the development and enrichment of new biofungicides for the control of rice sheath blight caused by *Rhizoctonia solani*. PCND 18-1 exhibited fungicidal activity against *R. solani*, showing an inhibition rate of 87.64%, with a 50% effective concentration (EC_{50}) 4.25 μg/mL, regression equation of Y = 0.7105x + 3.8428; and correlation coefficient of 0.9817, which are indicative of its potential as a natural biofungicide. PCND 18-1 also attenuated the pathogenicity of *R. solani*, which was concentration-dependent. Additionally, the action mode of PCND 18-1 against rice sheath blight (*R. solani*) was protective better than curative activity under greenhouse condition, as reflected by the corresponding EC_{50} values of 2.49 and 5.72 μg/mL. PCND 18-1 can translocate in rice with a low translocation capacity and exhibited a higher capacity for upward (root-leaf) translocation than for downward (leaf-root) translocation. Furthermore, PCND 18-1 demonstrated adhesion to leaves but poor tolerance against rain-washing. The optimal persistence period of PCND 18-1 on rice was 7 d. © 2018 Friends Science Publishers

Keywords: Carboxamidebiological characteristics; Derivative; Fungicidal activity; Phenazine-1-carboxamide; Plant pathogenic fungus; *Rhizoctonia solani*

Introduction

Rice sheath blight, which is caused by *Rhizoctonia solani*, is an important fungal disease that threatens rice production. Severe disease onset occurs when hot and humid environmental conditions are encountered in the late tillering and heading-grain filling stages. Severe cases of rice sheath blight can cause a 50% reduction in yield (Feng *et al.*, 2017). Current methods for controlling rice sheath blight mainly rely on agricultural control and chemical control. The agricultural control containing sclerotia salvage, diseased plants removal, adequate base-fertilizer applying, and appropriate field drying are labor-intensive, time-consuming, and poor efficiency. Though planting resistant cultivar is an effective control measure, the varieties with highly resistant to *R. solani* have been rare for a long time (Taheri and Tarighi, 2011; Moni *et al*., 2016). Therefore, a range of chemicals such as tebuconazole, boscalid, fludioxonil, and jinggangmycin, are always used during the outbreak of rice sheath blight, though the long term application of which not only causing the environmental pollution, ecological balance damaging, but also inducing fungicide resistance in pathogens and thereby reducing its effects (Wang *et al*., 2007; Ye *et al*., 2010; Tang *et al*., 2012; Ishaq *et al*., 2017). The biological control of rice sheath blight exhibits high efficiency, low toxicity, and no pollution and is thus considered as a promising alternative to chemical control. Current biological control techniques mainly consist of insect pest control, microbial pest control, bacterial control of pathogenic fungi, and fungal control of pathogenic fungi. The bacterial control of pathogenic fungi is one of the most important means that the bacteria include both the living bacteria and bacterial metabolites (Nagarajkumar *et al*., 2004; Huang *et al*., 2012). Because living bacteria are sometimes harmful to other organisms, the use of bacterial metabolites to control pathogenic fungi has become a major means of biological control (Joshi *et al*., 2008).

In our recent study, a bacterial strain with antagonistic activity against *R. solani*, designated as SU8, was screened from a rice-duck complex ecosystem, which was later identified as *Pseudomonas aeruginosa*. Further studies have

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clarified that the fungicidal component of strain SU8 is phenazine-1-carboxamide, which exhibits unstable control efficacy in field conditions (Zhang *et al.,* 2016). Therefore, we performed chemical modification of the structure of phenazine-1-carboxamide and obtained the derivative PCND 18-1 (chemical formula: $C_{23}H_{15}N_3O$; Molecular weight: 349.38; Fig. 1). To clarify the development potential of PCND 18-1, this study assessed its fungicidal activity, relevant biological characteristics against *R. solani*, and its contents in rice by high-performance liquid chromatography (HPLC), which in turn provides a theoretical basis and practical foundation for enriching new agents and developing novel bionic fungicides for the control of rice sheath blight.

Materials and Methods

Synthesis of PCND 18-1

The hydrolysis of phenazine-1-carboxamide produced phenazine-1-formic acid in acidic conditions. One millimole of phenazine-1-formic (0.2282 g), 1 mmol of 1-naphthylamine (0.1467 g), 2 mmol of three (trifluoroethoxyl) boron (0.4320 mL), and 2 mL acetonitrile were added into a 10-mL sample bottle, subjected to magnetic stirring, then allowed to react at 80°C for 24 h. Then 3 mL of trichloromethane and 0.5 mL of H_2O were added to the mixture to terminate the reaction. Exactly 150 mg of each Amberlyst A-26 (OH), Amberlyst 15, and Amberlite IRA743 were then added in succession and then stirred for 30 min at room temperature. Magnesium sulfate powder was added to the mixture to remove water. The resulting mixture was then filtered. The solid magnesium sulfate was washed thrice with trichloromethane. Rotary evaporation was then performed to yield PCND 18-1 (Fig. 2).

Preparation of Potato Dextrose Agar (PDA) Medium

Potatoes were washed and peeled, from which 200 g were weighed and cut into small cubes. The potato cubes were boiled in water for 20–30 min until these could be punctured using a glass rod. The potato and water were filtered through four layers of gauze, and the resulting filtrate was heated. According to the experimental requirements, 17–20 g of agar (Chemical reagents were purchased from Sinopharm Chemical Reagents, Shanghai City, China) was added, and the filtrate was continuously heated with constant stirring. When the agar was completely dissolved, glucose was added to the mixture, which was then thoroughly stirred. After slight cooling, water was added to the mixture to a final volume to 1,000 mL, and the medium was dispensed into conical flasks, stoppered, and wrapped up. The medium was placed in a sterilizer (LDZX-30E autoclave, Shenan Medical Instrument Factory, Shanghai City, China), and moist heat sterilization was performed at 121°C for 20 min. The flasks were then taken out, allowed to cool, and stored for later use (Fang, 1998).

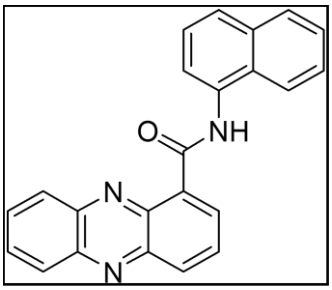

Fig. 1: Structural formula of PCND 18-1

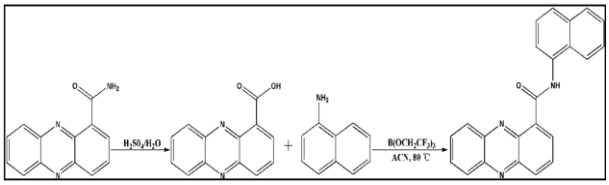

Fig. 2: Synthesis of PCND 18-1

Fungicidal Activity of PCND 18-1 against *R. solani*

The fungicidal activity of PCND 18-1 (96% PCND 18-1 was modified from phenazine-1-carboxamide in our laboratory) against *R. solani* (A wild-type sensitive strain was isolated, identified, and preserved by the Plant Pathology Laboratory at Hunan Agricultural University) was determined by measuring mycelial growth rate. PCND 18-1 was dissolved in an appropriate amount of acetone, diluted with sterile water to a working solution with a concentration of 1,000 μg/mL. The working solution was then mixed with PDA medium at a 1:9 volume ratio to obtain fungicide-containing plates with final fungicide concentrations of 0.01, 0.1, 1, 10 and 100 μg/mL. Fungicide-free plates were used as control. Mycelial discs were cut from the margin of 2-d-old *R. solani* colonies using a hole punch with a diameter of 5 mm and inoculated onto fungicide-containing plates, fungal side downward. Each treatment was repeated thrice. The inoculated plates were placed in a 26–28°C incubator (MPJ-250 incubator, Senxin Experimental Instrument Factory, Shanghai City, China). When the colony diameter reached two-thirds of the entire plate in the control, the colony diameter was measured by the cross method, and the inhibition rate in each treatment was calculated. The 50% effective concentration (EC_{50}), correlation coefficient and regression equation were calculated using DPS6.55 software (Hang Zhou, China).

Efficacy Assessment of PCND 18-1 on the *R. solani* Pathogenicity

R. solani mycelial discs were cultured on PDA plates at 26–28°C for 2 d and then immersed in a solution containing 2.5, 5.0, 10.0, 20.0 or 40.0 μg/mL PCND 18-1 for 30 s; a fungicide-free solution was used as control.

After air-drying, the mycelial discs were inoculated onto the sheath of the third-last leaf of disease-free rice plants ("Shenliangyou 1" was purchased from Hunan Longping Seed Industry Co., Ltd.). The plants were kept moist with absorbent cotton and cultured in a 26–28°C incubator (QHX-400B-III illumination incubator, Xinmiao Medical Equipment Manufacturing Factory, Shanghai City, China). Three pots were inoculated per treatment, with 30 plants per pot. Seven to nine days later, disease onset was assessed, and disease lesion diameter was measured. The inhibition rate was calculated as follows:

$$\text{Inhibition rate (\%)} = \frac{A-B}{C} \times 100$$

Where A is the lesion diameter of the control; B is the lesion diameter of each treatment; and C is the lesion diameter of the control - the diameter of mycelial cake.

Protective and Curative Activities of PCND 18-1 against rice Sheath Blight

Determination of protective activity: "Shenliangyou 1" seedlings showing uniform growth in a greenhouse were evenly sprayed with a solution containing 2.5, 5.0, 10.0, 20.0 or 40.0 μg/mL PCND 18-1; a fungicide-free solution was used as control. After 24 h of treatment, pre-cultured *R. solani* mycelial discs were embedded in the sheath of the third-last leaf of rice plants. Each treatment was repeated thrice, with 30 plants per treatment. The plants were grown at 28–32°C and kept moist in plastic bags. Nine days later, the lesion diameters of the inoculation sites were measured, as well as the disease lesion diameters. The inhibition rate was calculated using above mentioned equation. To determine curative activity, *R. solani* was inoculated as performed for the determination of protective activity, except that PCND 18-1 was sprayed 24 h after inoculation.

Preparation of Samples

Approximately 10 g of the roots, stems, and leaves of rice were collectively, respectively, external residues and dirt were removed, cut up using scissors, thoroughly mixed, ground in a mortar into a homogenate, and then stored at -20°C until analysis.

Extraction of Samples

Approximately 1 g of each sample was placed into a 100 mL conical bottle, to which1.5 mol/L hydrochloric acid was added, left to stand for 30 min, and the pH was adjusted 2–3. Then, 25 mL aliquots of each sample were transferred into centrifuge tubes; each sample was mixed with 25 mL of trichloromethane and then subjected to 10 min of ultrasonic extraction. The samples were centrifuged at 8,000 rpm for 3 min, the supernatant removed, and the pellet was subjected to trichloromethane extractions using anhydrous sodium sulfate. Approximately 30 mL of the resulting liquid from each sample was subjected to rotary evaporation at 40°C in a water bath to concentrate, mixed with methanol to a constant volume, passed through a 0.22 μm organic membrane filter to remove impurities, and then used in detection.

Application of PCND 18-1

The roots, leaves, and stems of rice leaves that were not treated with the agent were washed, cut, and then homogenized into a paste. Approximately 1 g of each sample was placed into a conical flask, which was then mixed with a quantitative standard methanol solution, respectively, and adjusted to concentrations of 0.4, 0.2, 0.1, 0.05, 0.025 μg/mL. Each process was repeated five times, including a blank.

Detection using HPLC

An Agilent 6470 HPLC system (Agilent Technologies, Germany) was used to detect PCND 18-1. The specific conditions were as follows: C18 stainless steel chromatographic column; flow phase: volume of methanol: volume of phosphate buffer, pH 7.5 = 60:40; Velocity: 1 mL/min; detection wavelength: 248 nm; and column temperature: 25°C. The sample quantity is 10 μL; Detection limit: 3 s/n.

Generation of Standard Curve of PCND 18-1

Approximately 1 mg of PCND 18-1 was weighed accurately using an electronic scale and dissolved in methyl alcohol, which was used as the stock solution with a concentration of 40 μg/mL. The stock solution was then diluted to various concentrations, namely, 4, 2, 1, 0.5 and 0.25 μg/mL. Each sample was then used in generating a standard curve, using each concentration as abscissa and peak area as the ordinate.

Systemic Translocation of PCND 18-1 in Rice

In the late tillering stage, disease-free rice plants were treated with 5 mL of the PCND 18-1 solution at various concentrations (2.5, 5.0, 10.0, 20.0 or 40.0 μg/mL) by root irrigation; a fungicide-free solution was used as control. After 24 h of treatment, pre-cultured *R. solani* mycelial discs were embedded into the sheath of the third-last leaf of rice plants. Each treatment was repeated thrice, with 30 plants per treatment. Nine days later, the diameter of the lesions was measured according to the lesion diameter of the inoculation site. The inhibition rate was calculated using Eq. (1), and the translocation capacity of the rice roots for PCND 18-1 was evaluated (Xu *et al*., 2014).

Rain-washing Tolerance of PCND 18-1 on Rice

At the late tillering stage, rice seedlings were sprayed with 2.5, 5.0, 10.0, 20.0 or 40.0 μg/mL PCND 18-1. Distilled water containing 0.1% (mass fraction) Tween-80 was used as control. A watering can was used to simulate rainfall and the seedlings were watered for 15 min at 1, 6, 12, 24 and 48 h after treatment, followed by *R. solani* inoculation. Each treatment was repeated thrice. Seven days later, the diameter of the disease lesions was measured. The inhibition rate was calculated using Eq. (1), and the control efficacy was evaluated.

Persistence of PCND 18-1 in Rice

PCND 18-1 at concentrations of 2.5, 5.0, 10.0, 20.0 and 40.0 μg/mL was evenly sprayed onto disease-free rice seedlings at the tillering stage; a fungicide-free solution was used as control. *R. solani* was inoculated as described in Subsection 1.6. At 1, 3, 5, 7 and 11 d after treatment, the diameter of the inoculation site and that of the disease lesions were measured. The inhibition rate was calculated using Eq. (1), and the persistence of PCND 18-1 on rice was evaluated.

Data Analysis

The raw data were first processed using Excel and then subjected to significant difference analysis by Duncan's multiple range test using DPS6.55 (Tang and Zhang, 2013).

Results

Structural Characterization of PCND 18-1

The 0.1858 g of light yellow PCND 18-1, which exhibit acicular crystallization, were obtained by chemical synthesis, with a yield of 53.18%. The structure of PCND 18-1 was confirmed by ^{1}H NMR, ^{13}C NMR, (Bruker AV-500 BioSpin, Switzerland; TMS as an internal standard; $CDCl_3$ solvent), and mass spectrum analyses. The main results were as follows. ^{1}H NMR (500 MHz, $CDCl_3$) δ 15.56 – 15.52 (m, 4H), 8.96 (dd, J = 7.0, 1.3 Hz, 71H), 8.63 – 8.54 (m, 7H), 8.54 – 8.46 (m, 74H), 8.46 – 8.28 (m, 85H), 8.28 – 8.11 (m, 79H), 8.11 – 7.56 (m, 356H), 7.52 (d, J = 7.8 Hz, 5H), 7.51 – 7.35 (m, 81H), 7.26 (dd, J = 14.8, 8.4 Hz, 83H), 6.76 (dt, J = 9.4, 4.7 Hz, 34H), 3.49 – 3.45 (m, 4H), 2.50 – 2.42 (m, 5H), 1.55 – 1.31 (m, 18H), 1.31 – 0.53 (m, 67H), 0.10 (s, 62H).) (Fig. 3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.88 (s), 144.04 (s), 143.32 (s), 142.01 (s), 139.98 (s), 139.77 (s), 137.35 (s), 135.06 (s), 134.26 (d, J = 19.5 Hz), 133.17 (s), 131.69 (s), 131.19 (s), 130.22 (s), 130.04 (s), 128.94 (s), 128.47 (s), 128.28 (s), 127.93 (s), 126.55 – 125.68 (m), 124.86 (d, J = 17.0 Hz), 123.63 (s), 121.56 (s), 120.75 (s), 118.92 (s), 109.69 (s), 77.31 (s), 77.06 (s), 76.80 (s), and 1.03 (s) (Fig. 4). MS (ESI) m/z 349.12 [M+H]+ (Fig. 5). The molecular formula of PCND 18-1 was $C_{23}H_{15}N_3O$. PCND 18-1 appeared as expected on the MS diagram and was confirmed by MS.

Table 1: Effect of PCND 18-1 against *R. solani*

Treatment (μg/mL)	Colony diameter (mm)			Average value (mm)	Inhibition (%)
100.00	13.60	13.50	14.00	13.70	87.64 ± 0.01[a]
10.00	28.60	28.70	28.80	28.70	59.96 ± 0.03[b]
1.00	48.40	48.70	47.80	48.30	23.80 ± 0.01[c]
0.10	55.60	56.20	56.20	56.00	9.59 ± 0.02[d]
0.01	58.00	58.40	58.50	58.30	5.35 ± 0.01[e]
CK	61.50	61.30	60.80	61.20	

Note: Numbers with different letters in the same column are significantly different according to Duncan's shortest significant range test. "CK" is the control group (Blank, the same as in the following tables)

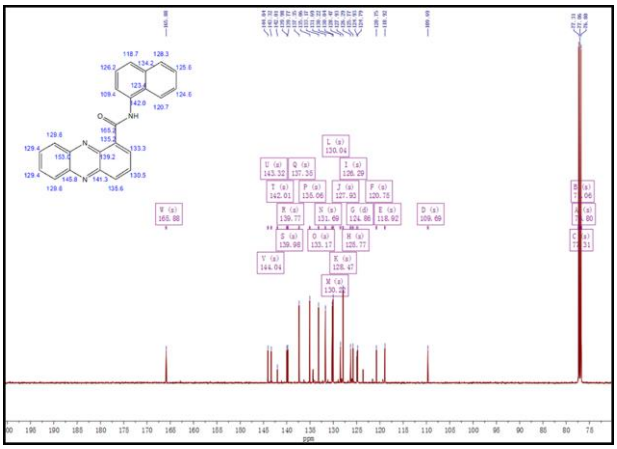

Fig. 3: C nuclear magnetic resonance spectrum of PCND 18-1

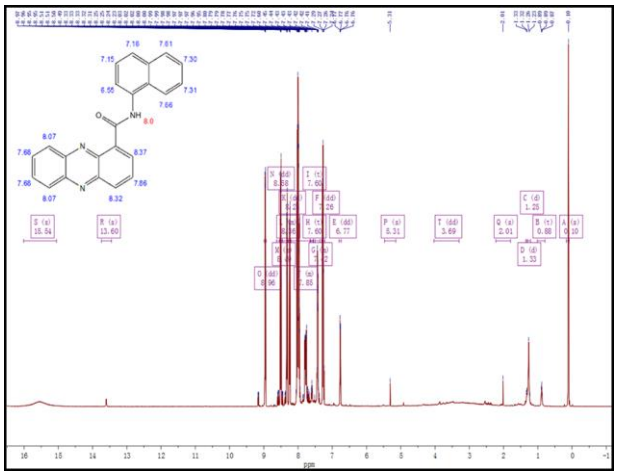

Fig. 4: Hydrogen nuclear magnetic resonance spectrum of PCND 18-1

Fungicidal Activity of PCND 18-1 against *R. solani*

Table 1 shows that PCND 18-1 exhibited fungicidal activity against *R. solani*. The higher the PCND concentration, the better the control efficacy. The best control efficacy was achieved when the concentration of PCND 18-1 was 100.00 μg/mL, with an inhibition rate of 87.64%.

The second-highest control efficacy was obtained with 10.00 μg/mL of PCND 18-1. The inhibition rate was below 50% for the remaining treatments. PCND 18-1 was highly toxic to *R. solani*, with an EC_{50} 4.25 μg/mL. The regression equation was Y = 0.7105x + 3.8428, and the correlation coefficient was 0.9817. These results suggested that PCND 18-1 can be used as a novel fungicide for the control of rice sheath blight.

Effect of PCND 18-1 on the Pathogenicity of *R. solani*

Table 2 shows that PCND 18-1 attenuated the pathogenicity of *R. solani*. The concentration of PCND 18-1 was inversely proportional to the pathogenicity of *R. solani*. When treated with 40.00 µg/mL PCND 18-1, the pathogenicity of *R. solani* was significantly attenuated, with an inhibition rate of 77%. The second highest control efficacy was obtained with 20.00 μg/mL of PCND 18-1. The inhibition rate was below 63% for the remaining treatments. These results indicate that PCND 18-1 attenuates the pathogenicity of *R. solani* and decreases the chance of pathogenic infection, thereby reducing disease onset in rice.

Protective and Curative Activities of PCND 18-1 against Rice Sheath Blight

Exploring the protective and curative activities of PCND 18-1 against *R. solani* is important to clarify the application of this agent before or after disease onset. We determined the protective activity of PCND 18-1 against rice sheath blight by application prior to inoculation. The results showed that PCND 18-1 has good control efficacy against *R. solani*, with an EC_{50} 2.49 μg/mL, a correlation coefficient of 0.9874, and a regression equation of Y = 0.8208x + 4.6743. Next, we determined the curative activity of PCND 18-1 by application after inoculation. The results showed that PCND 18-1 exhibited some control efficacy against *R. solani*, with an EC_{50} of 5.72 μg/mL, a correlation coefficient of 0.9434, and a regression equation of Y = 0.6305x + 4.5225 (Table 3). These results suggested that PCND 18-1 has better protective activity than curative activity.

Line Correlation

The samples of PCND 18-1 were diluted with methanol to a standard working solution at concentrations of 4, 2, 1, 0.5 and 0.25 μg/mL, respectively, using the concentration as the abscissa and peak area as the vertical axis to draw a standard working curve. The results show that when the retention time is 1.47 min, PCND 18-1 can be detected, indicating that the preset detection conditions meet the test requirements (Fig. 6). For the test concentration range, a good correlation (positive correlation) between the concentration of the sample and the peak area was observed, and the curve equation is Y = 913.40x - 100.70, R^2 = 0.9930, which could be used in subsequent studies.

Table 2: Effect of PCND 18-1 on *R. solani* pathogenicity (72 h)

Treatment (μg/mL)	Disease spot diameter (mm)			Average (mm)	value Inhibition (%)
2.50	19.28	19.22	19.28	19.26	44.94 ± 0.01[a]
5.00	16.60	16.65	16.70	16.65	55.02 ± 0.02[b]
10.00	14.81	14.71	14.76	14.76	62.32 ± 0.01[c]
20.00	11.17	11.17	11.11	11.15	76.25 ± 0.04[d]
40.00	10.86	10.87	10.82	10.85	77.41 ± 0.02[e]
CK	30.90	30.80	31.00	30.90	

Note: Numbers with different letters in the same column are significantly different according to Duncan's shortest significant range test

Table 3: Protective and curative activities of PCND 18-1 against *R. solani* (96 h)

Mode of action	Regression equation	Correlation coefficient	EC_{50} (μg/mL)
Protective activity	Y = 0.8208x + 4.6743	0.9874	2.4933
Curative activity	Y = 0.6305x + 4.5225	0.9434	5.7201

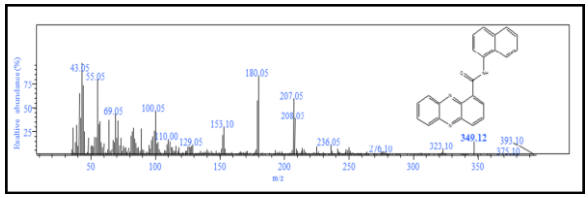

Fig. 5: Mass spectrum of PCND 18-1

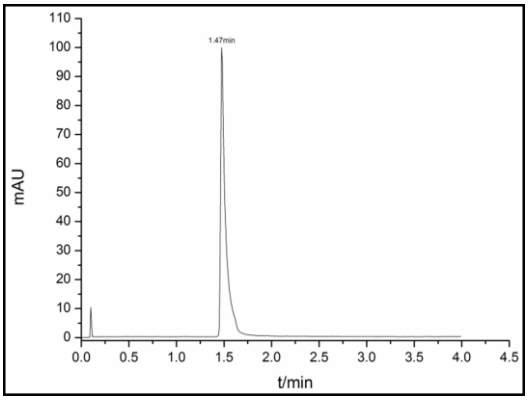

Fig. 6: Chromatogram of difenoconazole standard solution

Added Recovery Rate

PCND 18-1 was added to the roots, stems, and leaves as blank control, and the PCND 18-1 with a mass fraction of 0.025–0.40 μg/mg was used to extract, remove, and recover the samples according to the above experimental method (Table 4). The results showed that the average recoveries of PCND 18-1 in various substrates were between 88.01% and 96.22%, and the relative standard deviations were between 0.56% and 4.35%, which was in accordance with the requirements of pesticide residue analysis.

Table 4: Recovery of PCND 18-1 in rice leaves, stems, and roots

Sample	Fortification level / (μg /mg)	Recovery (%)						RSD /%
		1	2	3	4	5	Average	
Roots	0.025	93.22	91.46	94.61	92.88	94.17	93.27	1.23
	0.05	93.65	92.46	93.47	93.64	92.67	93.18	0.57
	0.1	92.13	91.64	94.65	94.18	95.46	93.61	1.65
	0.2	90.56	92.65	89.67	85.46	97.37	91.14	4.35
	0.4	96.34	95.84	95.47	96.66	96.79	96.22	0.56
Stems	0.025	90.31	88.56	85.77	86.96	88.44	88.01	1.72
	0.05	89.96	91.64	90.23	89.67	91.38	90.58	0.88
	0.1	91.32	92.56	90.34	88.74	90.41	90.67	1.41
	0.2	89.32	88.79	87.54	89.99	89.37	89.00	0.92
	0.4	92.35	89.74	82.64	85.79	89.94	88.09	3.85
Leaves	0.025	85.67	84.22	86.75	89.37	91.22	87.45	2.83
	0.05	88.79	91.26	90.37	87.79	92.34	90.11	1.84
	0.1	90.01	89.36	85.69	92.33	93.15	90.11	2.93
	0.2	91.16	89.99	92.34	93.65	92.25	91.88	1.38
	0.4	93.54	92.67	88.54	89.27	93.67	91.54	2.45

Translocation of PCND 18-1 in Rice

PCND 18-1 was applied to the roots of rice plants to measure its upward translocation. The results showed that the disease lesion diameter was smaller on the leaves of rice plants treated with PCND 18-1 than in the control, and an increase in PCND concentration increased resulted in more pronounced control efficacy. The best control efficacy was 92.73%, indicating that PCND 18-1 can be translocated from the root to the leaf of rice. Next, PCND 18-1 was smeared on the leaves of rice plants, and the control efficacy against rice sheath blight was observed. These results showed that PCND 18-1 has a certain degree of control efficacy against rice sheath blight, which was dose-dependent. The best control efficacy was 91.56%, indicating that PCND 18-1 has a higher capacity for upward translocation than for downward translocation in rice plants (Table 5).

It is possible to provide a theoretical basis for the rational development of PCND 18-1 dosage and the method of application. Further investigation showed that PCND 18-1 was more efficiently translocated from the roots to the leaves compared to that from the leaves to the roots, and the content of PCND 18-1 from the roots to leaves was 10.23–20.80 μg/mL. The highest PCND 18-1 content was observed in the roots (12.58–20.80 μg/mL), whereas the lowest was detected in the stems and leaves (Table 6). The PCND 18-1 can also be transported from leaves to roots, with the highest contents observed in the leaves, followed by the stems and the roots. The results showed that PCND 18-1 could control rice sheath blight by rooting or spraying.

Rain-washing Tolerance of PCND 18-1 on Rice Leaves

Table 7 shows that the control efficacy and rain-washing efficacy were better for higher concentrations of PCND 18-1. For treatment with 2.5 μg/mL of PCND 18-1, the inhibition rate varied between 20.45% and 26.64% after 15 min of simulated rain-washing for 1–48 h. For treatment with 40.0 μg/mL PCND 18-1, the inhibition rate ranged between 53.50% and 76.65% after 15 min of simulated rain-washing for 1–48 h, *i.e.*, 2.62–2.89 fold higher than the inhibition using the lowest-concentration treatment. Second, the control efficacy decreased with longer periods of simulated rain-washing. The best control efficacy was achieved after 15 min of simulated rain-washing for 1 h, with an inhibition rate within the range of 26.64–76.65%. The lowest control efficacy was observed after 15 min of simulated rain-washing for 48 h, with an inhibition rate within the range of 20.45–53.50%. These results suggested that PCND 18-1 has a weak tolerance to rain-washing despite its adhesion to rice leaves.

Resistance to rain erosion is an important indicator of drug stability, as it reflects its adhesion capacity and potential field application. Further investigation showed that PCND 18-1 is resistant to rain erosion. After exposure to rain, the highest PCND 18-1 contents were observed in the leaves of rice, followed by the stems and then the roots. A direct positive correlation between PCND 18-1 content in different plant parts and the drug concentration was observed (Table 8). The rhizomes of rice have the capacity to bind or absorb PCND 18-1, thereby improving its resistance to erosion.

Persistence of PCND 18-1 on Rice

We measured the lesion diameter of *R. solani* at different time intervals after the application of PCND 18-1 to rice seedlings. Both control efficacy and persistence improved with higher concentrations of PCND 18-1. After treatment with 2.5 μg/mL of PCND 18-1, the inhibition rate ranged between 23.10% and 41.16%; after treatment with 40.0 μg/mL of PCND 18-1, the inhibition rate ranged between 65.64% and 84.87%, *i.e.*, 2.06–2.84 fold higher than the lowest concentration. Second, both control efficacy and persistence of PCND 18-1 decreased over time after treatment.

Table 5: Systemic translocation of PCND 18-1 in rice

Treatment (μg/mL)	Inhibition rate (%)	
	Transmission from roots to leaves	Transmission from leaves to roots
2.50	57.33 ± 0.02^{e}	67.24 ± 0.01^{e}
5.00	78.27 ± 0.01^{d}	70.15 ± 0.03^{d}
10.00	81.08 ± 0.02^{c}	73.05 ± 0.01^{c}
20.00	89.28 ± 0.03^{b}	86.66 ± 0.04^{b}
40.00	92.73 ± 0.01^{a}	91.56 ± 0.02^{a}

Note: Numbers with different letters in the same column are significantly different according to Duncan's shortest significant range test

Table 6: Effect of different transmission conditions against PCND 18-1 contents in rice

Treatments (μg/mL)	Different transmission conditions content (μg/mL)					
	Transmission from roots to leaves			Transmission from leaves to roots		
	Roots	Stems	Leaves	Roots	Stems	Leaves
2.50	12.58 ± 0.40^{e}	9.41 ± 0.06^{e}	10.51 ± 0.39^{b}	6.96 ± 0.07^{e}	7.59 ± 0.17^{e}	9.84 ± 0.14^{e}
5.00	14.52 ± 0.32^{d}	10.23 ± 0.08^{d}	10.88 ± 0.19^{b}	7.38 ± 0.01^{d}	8.47 ± 0.20^{d}	11.67 ± 0.10^{d}
10.00	16.53 ± 0.16^{c}	12.58 ± 0.40^{c}	10.86 ± 0.02^{b}	8.46 ± 0.03^{c}	10.59 ± 0.10^{c}	13.34 ± 0.05^{c}
20.00	18.42 ± 0.39^{b}	14.49 ± 0.10^{b}	11.58 ± 0.49^{a}	9.73 ± 0.17^{b}	11.38 ± 0.3^{2b}	15.38 ± 0.44^{b}
40.00	20.80 ± 0.22^{a}	15.69 ± 0.35^{a}	11.77 ± 0.37^{a}	10.73 ± 0.36^{a}	12.58 ± 0.10^{a}	16.70 ± 0.38^{a}

Note: Numbers with different letters in the same column are significantly different according to Duncan's shortest significant range test

Table 7: Rain-washing tolerance of PCND 18-1

Treatment (μg/mL)	Inhibition rate (%)				
	1 h	6 h	12 h	24 h	48 h
2.50	26.64 ± 0.02^{e}	24.77 ± 0.01^{e}	24.54 ± 0.01^{e}	21.66 ± 0.02^{d}	20.45 ± 0.03^{d}
5.00	37.29 ± 0.03^{d}	38.45 ± 0.01^{d}	39.23 ± 0.01^{c}	29.38 ± 0.01^{c}	29.86 ± 0.02^{c}
10.00	54.21 ± 0.03^{c}	46.81 ± 0.01^{c}	30.21 ± 0.02^{d}	30.08 ± 0.03^{c}	30.00 ± 0.03^{c}
20.00	70.61 ± 0.03^{b}	68.84 ± 0.02^{b}	52.31 ± 0.01^{b}	49.51 ± 0.01^{b}	46.99 ± 0.02^{b}
40.00	76.65 ± 0.01^{a}	73.40 ± 0.01^{a}	66.03 ± 0.01^{a}	63.23 ± 0.02^{a}	53.50 ± 0.01^{a}

Note: Numbers with different letters in the same column are significantly different according to Duncan's shortest significant range test

Table 8: Effect of rain against PCND 18-1 content in rice (48 h)

Treatment (μg/mL)	Different parts content (μg/mL)		
	Roots	Stems	Leaves
2.50	4.15 ± 0.07^{e}	4.24 ± 0.12^{e}	5.73 ± 0.08^{d}
5.00	4.31 ± 0.05^{d}	6.25 ± 0.10^{d}	7.54 ± 0.20^{c}
10.00	6.73 ± 0.07^{c}	8.49 ± 0.15^{c}	7.80 ± 0.14^{c}
20.00	8.05 ± 0.07^{b}	9.12 ± 0.03^{b}	9.42 ± 0.07^{b}
40.00	8.44 ± 0.11^{a}	9.34 ± 0.04^{a}	11.49 ± 0.21^{a}

Note: Numbers with different letters in the same column are significantly different according to Duncan's shortest significant range test

At PCND 18-1 concentrations of 2.5 to 5.0 μg/mL, the inhibition rate first increased and then decreased. At PCND 18-1 concentrations of 10.0 to 40.0 μg/mL, the inhibition rate gradually decreased (Table 9). These results illustrated that PCND 18-1 has a certain degree of persistence, although the persistence period is relatively short.

Pesticide duration not only influences the shelf life of a product, but also affects drug efficacy. Therefore, persistence duration plays an important role in creating of novel pesticides. Further investigation showed that PCND 18-1 has a certain degree of persistence. The highest PCND 18-1 content was observed in the leaves of the rice plants, followed by the stems and the roots (Table 10). Higher the concentration of PCND 18-1 in different parts of rice, the higher the content, thereby higher the amount of application, higher is its durability.

Discussion

In recent years, a number of studies have suggested that natural phenazines possess broad-spectrum fungicidal activity and exhibit the potential for developing biofungicides (Jin *et al.*, 2015). Phenazine-1-carboxylic acid exhibited strong fungicidal activity against *R. solani*, with an IC_{50} 0.068 μM/mL (Ye *et al.*, 2010). The present study showed that the phenazine-derived PCND 18-1 had a high toxicity against *R. solani*, with an EC_{50} 4.25 μg/mL, which is better than the toxicity of jinggangmycin reported by Chen (EC_{50} = 96.29 μg/mL). The EC_{50} of PCND 18-1 against rice sheath blight is 0.04-fold higher than that of a previous study (Chen *et al.*, 2013). However, the toxicity of PCND 18-1 against *R. solani* is lower than the toxicity of some common chemical fungicides such as mepronil (EC_{50}=0.09 μg/mL

Table 9: Persistence of PCND 18-1 against *R. solani*

Treatment (μg/mL)	Inhibition rate at different inoculation time points after treatment				
	1 d	3 d	5 d	7 d	11 d
2.50	23.10 ± 0.02^{e}	36.69 ± 0.03^{e}	38.78 ± 0.02^{e}	41.16 ± 0.01^{d}	32.80 ± 0.01^{e}
5.00	34.21 ± 0.01^{d}	45.59 ± 0.02^{d}	49.47 ± 0.01^{d}	43.11 ± 0.01^{d}	39.61 ± 0.03^{d}
10.00	51.29 ± 0.01^{c}	51.18 ± 0.01^{c}	44.09 ± 0.03^{c}	47.30 ± 0.03^{c}	43.91 ± 0.01^{c}
20.00	70.74 ± 0.02^{b}	68.79 ± 0.02^{b}	61.40 ± 0.01^{b}	61.68 ± 0.02^{b}	51.92 ± 0.02^{b}
40.00	84.87 ± 0.04^{a}	79.92 ± 0.01^{a}	74.24 ± 0.01^{a}	71.61 ± 0.03^{a}	65.64 ± 0.03^{a}

Note: Numbers with different letters in the same column are significantly different according to Duncan's shortest significant range test

Table 10: Effect of persistence against PCND 18-1 content in rice (11 d)

Treatment (μg/mL)	Different parts content (μg/mL)		
	Roots	Stems	Leaves
2.50	1.24 ± 0.12^{e}	1.91 ± 0.04^{d}	2.22 ± 0.04^{d}
5.00	1.92 ± 0.08^{d}	3.28 ± 0.24^{c}	3.32 ± 0.08^{c}
10.00	2.15 ± 0.04^{c}	3.50 ± 0.20^{c}	4.38 ± 0.06^{b}
20.00	2.37 ± 0.12^{b}	4.87 ± 0.11^{b}	4.67 ± 0.45^{b}
40.00	4.2 ± 0.08^{a}	5.62 ± 0.07^{a}	6.30 ± 0.17^{a}

Note: Numbers with different letters in the same column are significantly different according to Duncan's shortest significant range test

(Zhang *et al.*, 2009), azoxystrobin (EC_{50}=0.08 μg/mL, (Jin *et al.*, 2009), and fluxapyroxad (EC_{50}=0.05 μg/mL (Chen *et al.*, 2014). Tang showed that propiconazole has the highest toxicity to *R. solani*, with an EC_{50} 0.045 μg/mL (Tang *et al.*, 2012). However, some studies have shown that boscalid shows inconsistencies in its toxicity against *R. solani*. Chen reported that boscalid has an EC_{50} 2.12 μg/mL against *R. solani*, whereas Zhang found that boscalid has an EC_{50} 2.04 μg/mL against the same pathogen (Zhang *et al.*, 2010; Chen *et al.*, 2014). Chen indicated that boscalid has the highest toxicity to *R. solani*, with an EC_{50} 1.07 μg/mL (Chen *et al.*, 2013). Additionally, Zhang reported 5-hydroxyl-5-methyl-2-hexenoic acid to have an IC_{50} 61.61 μg/mL against rice sheath blight; however, their method is different from ours in this study, and the toxicity of 5-hydroxyl-5-methyl-2-hexenoic acid to *R. solani* needs to be further assessed (Zhang *et al.*, 2010). Moreover, compounding and synergists can be used to further improve the toxicity of PCND 18-1 against *R. solani*, but their effectiveness also needs to be verified.

Disease onset increases in severity as the pathogenicity of a plant pathogenic fungus increases. The results of this study showed that PCND 18-1 attenuates the pathogenicity of *R. solani*, which may be related to the inhibition of cell wall-degrading enzyme activity in *R. solani* by this compound.

Our results showed that PCND 18-1 exhibited higher protective activity than curative activity. Thus, PCND 18-1 can achieve its ideal control effect when it is used prior to disease onset. However, whether PCND 18-1 imparts a fungicidal or fungistatic effect needs further determination. Thus far, the killing effect of new fungicides has not been mentioned in relevant studies. In addition, when investigating the protective and curative activities of new fungicides, a number of studies did not clear indicate which activity is superior. For example, Chen believed that fluxapyroxad has equivalent protective and curative activities against *R. solani*; thus, fluxapyroxad can effectively control the onset of sheath blight whether applied before or after disease onset. Tang speculated that propiconazole has good protective and curative activities against rice sheath blight but did not clarify which activity is more significant, leading to difficulties in its application (Tang *et al*., 2012). Early application can effectively control the onset of rice sheath blight but might also introduce certain issues, for the early application of chemical agents can not only increase the risk of fungicidal residue accumulation in soil, but also cause excessive application or wastage of pesticides once the rice sheath blight is not severe in the current season. Therefore, the application of PCND 18-1 should be based on various factors and actual production conditions to effectively prevent disease.

Generally, the translocation of pesticides in plants is a comprehensive topic involving the compound, plant and application method. The mode of translocation not only influences the expression of the biological activity of new pesticides but also has an important impact on their scope of action and application technique. Biological and tracer methods were most commonly used methods in exploration of internal absorption and distribution of new compounds, although these had disadvantages of being time-consuming, low in precision, and causing radioactive pollution. Recently chromatographic methods attract more attention because of its accuracy, sensitivity, and rapid analysis. In this study, the biodegradation and distribution of PCND 18-1 in rice were studied using a combination of biological techniques and chromatography. The two methods are complementary, as well as quantitatively clarified the distribution and content of the compound in rice, thereby providing a theoretical basis for further development of novel pesticides. This study showed that PCND 18-1 was translocated in rice plants, although with low translocation capacity. The conduction of PCND 18-1 from roots to leaves was superior to that from leaves to roots, and the higher the concentration of PCND

18-1, the higher the content in different parts of rice. Additional studies on the compound should be performed, including its distribution at different growth stages and rice varieties. Additionally, root irrigation might not be an effective application method, whereas foliar spraying may achieve good efficacy.

The low efficiency of pesticides is partially due to rain-washing. This study showed that PCND 18-1 adheres to the surface of rice leaves, although its rain-washing tolerance is relatively poor. Moreover, within the same period of time, control efficacy and rain-washing tolerance improved using higher concentrations of PCND 18-1. The rain-washing tolerance of this compound may be related to its deposition amount. Prolonged rain-washing decreased its efficacy in controlling disease. This phenomenon may be related to the loose adhesion of the compound onto the surface of rice plants or to other factors such as the rough surface of rice and/or the poor adsorption capacity of the compound. Therefore, when using PCND 18-1, we recommended that an adhesive be added to improve efficacy, as described by a previous study.

Furthermore, our results showed that when PCND 18-1 was applied at concentrations of 200–400 μg/mL, the best persistence period for the control of rice sheath blight was 7 d; after 7 d, the control efficacy was significantly reduced. These results indicate that PCND 18-1 has a short persistence period, possibly because it is easily degradable or tends to form new compounds. The short persistence period may also be related to its low translocation capability. Due to the limited research time and resources, we have not yet investigated the mechanism of action of PCND 18-1 against *R. solani* or performed stability tests (*e.g*., temperature, light and pH) or field demonstration studies, which will be performed in a further investigation.

Conclusion

PCND 18-1 exhibited fungicidal activity against *R. solani*. PCND 18-1 also attenuated the pathogenicity of *R. solani*, which was concentration-dependent. The action mode of PCND 18-1 against rice sheath blight (*R. solani*) was protective better than curative activity under greenhouse condition. PCND 18-1 can translocate in rice with a low translocation capacity and exhibited a higher capacity for upward (root-leaf) translocation than for downward (leaf-root) translocation. PCND 18-1 demonstrated adhesion to leaves but poor tolerance against rain-washing. The optimal persistence period of PCND 18-1 on rice was 7 d.

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