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Pseudomonas fluorescens RB5 as a Biocontrol Strain for Controlling Wheat Sheath Blight Caused by *Rhizoctonia cerealis*

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Abstract: Wheat sheath blight is a soil-borne fungal disease caused by *Rhizoctonia cerealis* and is a serious threat to wheat worldwide. A microbial fungicide is a promising alternative to a chemical fungicide for wheat disease control. In this study, strain RB5 against *R. cerealis* was isolated from wheat rhizosphere soil, which was identified as *Pseudomonas fluorescens* according to physiological, biochemical, and 16S rRNA gene sequence analyses. For improving the antifungal activity of RB5, the response surface methodology (RSM) was used to optimize the culture conditions for strain RB5, and the optimal culture conditions are 8.7 g/L of cassava, 5.2 g/L of soybean meal, pH 6.8, a 218 r/min speed, a 31.5 °C temperature, and 54 h of culture time. The inhibition rate of the culture filtrate obtained under this culture condition was up to 79.06%. The investigation of action mechanism showed strain RB5 could produce protease, chitinase, and siderophore, and its culture filtrate disrupted the mycelial morphology and inhibited the activities of three cell-wall-degrading enzymes of *R. cerealis*. Furthermore, the pot experiment exhibited that RB5 significantly controlled the wheat sheath blight with an efficacy of 71.22%. The evaluation of toxicological safety on an animal indicated that the culture filtrate was safe on mice. Overall, the culture filtrate of RB5 is a very promising microbial fungicide for the control of wheat sheath blight.

Keywords: *Rhizoctonia cerealis*; Pseudomonas fluorescens RB5; culture filtrate; biological control; animal toxicological safety

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1. Introduction

As one of the most widely planted crops, wheat (*Triticum aestivum* L.) is a staple food for more than 35 percent of the world's population [1]. Therefore, ensuring high and stable yields in wheat production is of great significance. As wheat grows and develops, it always suffers many biotic stresses, which damage crops, inhibit their growth and reduce the grain yield [2]. Among these biotic stresses, *Rhizoctonia cerealis* is a notorious pathogenic fungi causing wheat sheath blight [3]. *R. cerealis* disrupts the wheat mechanical and conductive tissues of the stem and other parts, resulting in the necrosis of stem walls, and ultimately hindering the transportation of nutrients and water, leading to the absence of grains in wheat spikes [4]. Moreover, the sheath blight currently occurs in more than 100 countries in the Asia-Pacific region, the Americas, the Middle East, and Africa [5–7]. And every year in China, wheat sheath blight causes serious yield and economic losses [3,7].

Nowadays, measures to control wheat diseases have been widely developed and practiced, including field management and chemical and biological control. In agricultural production systems, field management includes ploughing activities, fertilizer type

and timing of application, and irrigation, as well as soil type and climatic conditions [8]. However, inappropriate field management can increase the probability of disease and pest infection, which affect the yield and quality of wheat [9]. Moreover, the excessive use of fungicides will pollute the environment, result in the fungicide resistance of pathogens, and pose a serious threat to human health [10]. Therefore, as a safe, eco-friendly, efficient, and low-cost method, biological control has become a promising alternative approach for controlling wheat sheath blight in sustainable agriculture development [11]. Biocontrol microorganisms or secondary metabolites for preventing and controlling plant diseases have been reported [12]. Some bacteria are used as biocontrol agents against a crop, such as *Pseudomonas* spp., *Bacillus* spp., *Enterobacter* spp., *Agrobacterium* spp., and so on [13,14]. Sriwati et al. [15] reported that endophytic bacteria could control *Fusarium oxysporum* and promote tomato plants' growth, revealing its potential for the development of a biocontrol agent. Zhao et al. [16] found that *Alternaria*, *Ascochyta*, *Botryosphaeria*, and *Talaromyces* could inhibit *R. cerealis*, and *Talaromyces assiutensis* R-03 was determined as a promising agent.

At present, most reports on the control of wheat sheath blight involve *Bacillus* sp. [17,18]. Yi et al. proposed *Bacillus subtilis* XZ18-3 that inhibited *R. cerealis* mycelia growth through a culture filtrate [19]. Deng et al. evaluated that *Bacillus amyloliquefaciens* PEBA20 could be taken as a biocontrol agent against *R. cerealis* [20]. Peng et al. [14] proposed the application of *Bacillus subtilis* NJ-18 with 20% Flutolanil for the control of wheat sheath blight with an efficacy of 68.9%. Among biocontrol bacteria, *P. fluorescens* have many desirable characteristics in terms of activity, such as antifungal, phosphate solubilization, siderophore production, hydrogen cyanide, hydrolase, ammonia, and plant growth regulation activities, which allow them to be used as a plant growth promoter and biological control agent [21]. For instance, *Pseudomonas* significantly inhibited the growth of *R. solani* AG1-IA in vitro and reduced the disease symptoms at the booting stage of the rice plant as well [22]. *Pseudomonas fluorescens* strain 2–79 combined with pencycuron was used for the integrated control of root rot disease [23].

Although *P. fluorescens* has received some attention, it is far inferior to *Bacillus* spp. For obtaining more effective bacteria antagonistic to *R. cerealis*, the present study aimed to screen and identify *P. fluorescens* RB5, optimizing the culture conditions of strain RB5, and investigate its antifungal mechanism and biocontrol efficacy. Furthermore, an animal safety evaluation for *P. fluorescens* RB5 was performed and showed that RB5 could be developed as safe and effective biocontrol fungicides to control wheat sheath blight.

2. Materials and Methods

2.1. Materials

Soil samples were collected from wheat rhizosphere soil in Zhengzhou, Henan Province. *R. cerealis* was stored in a laboratory of the School of Biological Engineering, Henan University of Technology. Wheat seeds (Yumai 49) were purchased from Henan Academy of Agricultural Sciences. KM mice (SCXK 2015-0004) (SPF grade, 18~22 g, good health) were purchased from the Henan experimental animal center.

2.2. Isolation and Screening of Strains against R. cerealis

Antagonistic bacteria were isolated following the method of Yi et al. [24] with minor modifications. Briefly, 10.0 g of soil was added into a 500 mL conical flask with 90 mL of sterile water and glass beads, thoroughly stirring for 30 min to obtain a 0.1 g/mL soil suspension. The suspensions were diluted with the 10 × gradient dilution method, and 20 µL of aliquots were spread on Luria–Bertani (LB) (5 g of yeast extract, 10 g of peptone, 10 g of NaCl, 1 L of sterile water, pH 7) plates and incubated for 48 h. Colonies that appeared on LB agar plates were scribed 4-5 times until a single pure colony appeared. The isolated bacteria were labeled with numbers.

Bacteria against *R. cerealis* were preliminarily screened with the confrontation culture method [25]. Five millimeters of the pathogenic fungus plug was inoculated on the center of the PDA (200 g of potato, 20 g of glucose, 15 g of agar, 1 L of sterile water, pH 7) plate.

Labeled bacteria were inoculated at a distance of 2.5 cm from the fungal plug. Plates only inoculated with the pathogenic fungus plug were used as a control and all plates were incubated at 28 °C for 5 days. Strain RB5 with wider antimicrobial bands was screened out.

2.3. Identification of Antagonistic Strain RB5

The colony morphology (size, color, and transparency) of strain RB5 was observed on the LB plate. Physiological and biochemical tests including a nitrate reduction, a glucose reaction, citrate, indole, amylolysis, a contact enzyme, methyl, and a Voges Proskauer (VP) test were carried out according to *Berger's Manual of Systematic Bacteriology* [26]. The genomic DNA of strain RB5 was prepared with the modified alkaline lysis method of Yi et al. [24]. A 16S rRNA gene sequence was amplified with the universal bacterial primers 16SF (5′-AGAGTTTGATCATGGCTCAG-3′) and 16SR (5′-TGAAGTCGTAACAAGGTAACCGT-3′). The conditions for PCR were as follows: 94 °C for 4.0 min, then 32 cycles of denaturation at 94 °C for 50 s, annealing at 53 °C for 1.0 min, and extension at 72 °C for 40 s, followed with a final extension at 72 °C for 8 min. PCR amplification products were verified with 1.0% agarose gel electrophoresis and sequenced by Biomed Co., Ltd. (Beijing, China). Then, 16S rRNA gene sequences were analyzed on the NCBI website (National Center for Biotechnology Information, Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov/Blast (accessed on 4 February 2010) and used for constructing for the phylogenetic tree with the neighbor-joining method by MEGA 7.0 [27].

2.4. Detection of Hydrolase and Siderophore

According to the method of Makhdoumi Kakhki et al. [28], cellulase, chitinase, protease, and pectinase produced from strain RB5 were detected using a carboxy methyl cellulose medium, colloidal chitin medium, skim milk medium, and pectin medium, respectively. Strain RB5 was inoculated on the detected plates for hydrolase and incubated at 40 °C for 21 days, and the clear zones were observed visually. The siderophore production was analyzed using color changes after 2 days of the culture on the CAS agar plate using the method described by Schwyn et al. [29].

2.5. Optimizing the Culture Conditions for RB5 with Response Surface Methodology (RSM)

2.5.1. Single-Factor Method for Determining the Optimal Components

In order to improve the inhibition rate of RB5 against *R. cerealis*, culture conditions of strain RB5 were optimized concerning different culture conditions such as N and C sources. Nitrogen sources (yeast paste, soybean flour, peptone, soybean meal) and carbon sources (glucose, sucrose, glycerin, cassava) at a 0.1% concentration were used separately to observe their effects on the inhibition of the culture filtrate under the optimized conditions.

The culture of strain RB5 was centrifuged at 4 °C with 10,000 rpm for 10 min and filtrated with a 0.22 μm filter for preparing a cell-free culture filtrate. Then, 10 mL of the culture filtrate was mixed with 10 mL of the PDA medium and poured onto a flat plate. In total, 5 mm of the pathogenic fungus plug was inoculated on the center of the PDA plate. Sterile water without the culture filtrate was taken as a control and all experiments were conducted three times. After incubation at 25 °C for 7 d, the mycelial diameter was measured for calculating the inhibition rate with the following formula [18].

$$\text{Inhibition rate (\%)} = [(C - T)/C] \times 100 \quad (1)$$

where C represents the radial diameter of the pathogenic mycelia in the control plate, and T represents the radial diameter of the pathogenic mycelia in the treatment group.

2.5.2. Screening of Significant Variables with Placket–Burman Design

The Placket–Burman (PB) test is a powerful technique used to screen and evaluate important variables that affect a response. The model significantly reduces the number of experiments required to effectively achieve experimental objectives [30]. In this test, process variable optimization was performed using the PB design by Design-Expert 7.0

(DX7). Seven independent impact factors (cassava, soybean meal, temperature, pH, speed, amount of inoculation, and culture time) were investigated to identify the variables that significantly affect the antimicrobial activity. Each variable was divided into two levels: "1" and "−1". The inhibition rate (Y) was set as the response value. The factors and levels are shown in Table 1. The tests were conducted three times.

Table 1. Seven factors and two levels in the Placket–Burman design.

Factors	Numbers	−1	1
Cassava/(g/L)	A	6.0	12.0
Soybean meal/(g/L)	B	4.0	9.0
Temperature/°C	C	28.0	35.0
pH	D	6.8	8.5
Speed/(r/min)	E	160.0	240.0
Amount of inoculation/%	F	2.0	2.5
Culture time/h	G	48.0	60.0

2.5.3. Optimization of the Culture Conditions with Box–Behnken Design

Based on the results from the PB design test, a Box–Behnken experiment was performed to determine the optimal conditions using three independent factors of cassava (A), soybean meal (B), and speed (E), with three different levels (high (+1), medium (0), and low (−1)) (Table 2). The regression equation of the response values of the 15 test points was analyzed with Design-Expert 7.0 to determine the optimal culture conditions for strain RB5. For each variable, quadratic models were represented as a contour line map (3D), and response surface curves were generated using Design Expert 7.0. The process was repeated three times.

Table 2. Factors and levels for Box–Behnken design.

Factors	Numbers	−1	0	1
Cassava/(g/L)	A	8.0	9.0	10.0
Soybean meal/(g/L)	B	5.0	6.0	7.0
Speed/(r/min)	E	180	200	220

2.6. Detection of Antifungal Activity of RB5 Culture Filtrate

The antifungal activities of the culture filtrate of RB5 were examined with the mycelia growth rate method [31]. Briefly, strain RB5 was added to 100 mL of the LB broth in the flasks and shaken at 28 °C for 12 h with 200 r/min. Then, a 4% (v/v) aliquot of the RB5 suspension was inoculated into 200 mL of the LB broth and incubated at 28 °C and 180 r/min for 48 h. The cell-free culture filtrate was prepared as described in Section 2.5.1. Then, 2, 3, 4, 5, 8, 10, 15, and 20 mL of the culture filtrate were mixed with sterile water, and made the total volume of 20 mL for preparing the culture filtrate with different concentrations (10%, 15%, 20%, 25%, 40%, 50%, 75%, 100%). In total, 10 mL of the culture filtrate with different concentrations was mixed with 10 mL of the PDA medium and poured onto a flat plate, respectively. Sterile water without the culture filtrate was taken as a control and all experiments were conducted three times. The culture condition and inhibition rate follow Section 2.5.1.

2.7. Effect on Mycelial Morphology of R. cerealis Treated with Culture Filtrate

The culture filtrate was prepared as described in Section 2.5.1, and its effect on the mycelia morphology was observed under an optical microscope. The pathogenic fungi were incubated in a potato glucose broth (PDB) containing a 50% culture filtrate at 25 °C for 24 h on a rotary shaker with 180 rpm. PDB only contained a pathogen without the culture filtrate was set as a control. Then, the morphology of *R. cerealis* was observed under an optical microscope. This process was repeated three times.

2.8. Assay of Cell-Wall-Degrading Enzyme Activities of R. cerealis Treated with Culture Filtrate

2.8.1. Enzyme Extraction and Assay

An enzyme extract was prepared with the method of Marcus et al. [32]. Briefly, 0.5 g of mycelia of *R. cerealis* was added to 60 mL of a Marcus Enzyme Purification Medium and mixed with a different culture filtrate (0%, 40%, 50%, 75%, and 100%), respectively. The mixture was incubated on a shaker with 145 rpm at 28 °C for 8 days. Then, the culture was centrifuged at 4 °C with 10,000 rpm for 10 min and the supernatant was added (cold acetone (80:20, *v*/*v*)). The precipitate was removed after centrifugation with 12,000× *g* rpm for 30 min at 0 °C. Then, the collected supernatant was subjected to acetone precipitation. The precipitate was dissolved in 60 mL of an acetic-acid–sodium-acetate buffer (50 mmol/L, pH 5.0) as an enzyme extract for further tests. The detections of each enzyme activity were performed in triplicates. The protein concentration was determined using Lowry's method [33]. The release of reducing sugar was measured using the dinitrosalicylic acid (DNS) method [34].

2.8.2. Determination of Polygalacturonase (PG) Activity

PG activity was determined with the method of Figueroa et al. [35] with minor modifications. In brief, 1 mL of the enzyme extract, 1 mL of the acetic-acid–sodium-acetate buffer (50 mmol/L, pH 5.0), and 1 mL of the substrate (1% sodium pectin, prepared with 50 mmol/L of the acetic-acid–sodium-acetate buffer, pH 5.0) were mixed and reacted in a 30 °C water bath for 30 min. Next, 2.0 mL of the DNS was added into the mixture and incubated in a boiling water bath for 5 min. Then, the light absorption value of reactants was measured at 520 nm. In blank control tubes, the enzymatic extract was replaced by the acid-sodium-acetate buffer. PG activity was expressed as μg reducing sugar mg^{-1} min^{-1}.

2.8.3. Determination of Polymethyl Glacturonase (PMG) Activity

PMG activity was determined with the method of Denou et al. with minor modifications [36]. Briefly, 1 mL of the enzyme extract, 1 mL of a citrate buffer (50 mmol/L, pH 4.5), and 1 mL of the substrate (1% sodium pectin, prepared with 50 mmol/L of the citrate buffer, pH 4.5) were mixed and reacted in a 30 °C water bath for 30 min. Next, 2.0 mL of the DNS was added into the mixture and incubated in a boiling water bath for 5 min. Then, the light absorption value of reactants was measured at 520 nm. The citrate buffer replacing the enzymatic extract was used in the blank control tubes. PMG activity was expressed as μg reducing sugar mg^{-1} min^{-1}.

2.8.4. Determination of Pectin Methyl Trans Eliminase (PMTE) Activity

PMTE activity was determined as previously described with some modifications [37]. In total, 1 mL of the enzyme extract, 1 mL of a Gly-NaOH buffer (50 mmol/L, pH 9.0), 1 mL of the substrate (1% sodium pectin, prepared with 50 mmol/L of the Gly-NaOH buffer, pH 9.0), 1.0 mL of $CaCl_2$ (13 mmol/L), and 1.0 mL of sterile water were mixed and reacted in a 30 °C water bath for 30 min. The ultraviolet absorptive value of reactants was measured at 232 nm. In the blank control tubes, the enzymatic extract was replaced by sterile water. PMTE activity was expressed as μM unsaturated aldehydes mg^{-1} min^{-1}.

2.9. Pot Control Tests

The surface of 49 seeds of wheat Yumai was disinfected with 0.5% NaClO. Sterilized seeds were put in a Petri dish and inoculated with *R. cerealis*, and then were incubated at 25 °C for 3 to 5 d. The seeds were sown in pots (25 cm in diameter) filled with soil (peat/vermiculite/nutrition soil = 1:1:2). The pot experiment was set up using a completely randomized design with 4 different treatments and conducted at 26 to 30 °C, with relative humidity ranging from 60 to 70%, and with the light/dark (L:D) ratio of 12 h:12 h. At the two-leaf stage of wheat, 20 mL of different concentrations of the strain RB5 suspension (1×10^5, 1×10^7, and 1×10^9) and carbendazim were sprayed separately onto the wheat

seedlings. The sterile water was set as a control. Each treatment had three replicates with at least 50 wheat plants in each replicate.

After infecting with the pathogen in the control group, disease incidence was assessed according to the five (0–4) scoring standard. Level 0 indicates healthy plants, level 1 indicates that the incidence is less than or equal to 25%, level 2 indicates that the incidence is more than 25% to 50%, level 3 indicates that the incidence is more than 50% to 75%, and level 4 indicates that the incidence is more than 75%. The disease incidence rate (DIR), disease index (DI), and control efficacy (CE) were calculated through the formulas of Yi et al. [24].

2.10. Evaluate the Safety of RB5 Culture Filtrate on Animals

The culture filtrates were concentrated, ground, and sterilized to make powder. In total, 0.5 g of powder was dissolved in 9.5 mL of sterile water to obtain a 5% concentrated solution and used for animal toxicity evaluation. Mice were selected as experimental animals and their raising conditions were carried out according to the method of Wang et al. [38]. Briefly, healthy KM mice were kept in a room maintained at a temperature of 22 °C, with a relative humidity of 40 to 70% and 12 h:12 h light/dark cycle. After 7 days of adaptation, KM mice were divided into two groups, each group containing ten males and ten females. The mice in the treatment group were administered 20 mL/kg·bw of culture filtrates with oral gavage every day for 7 consecutive days. The control group of mice was administered the same amount of physiological saline. After gavage, the individuals of each group of mice were weighed at the beginning and end, respectively. Observations of any changes in skin, eyes, and fur were carried out and recorded every day [39,40]. The serological physiological indexes of alanine transaminase (ALT), aspartate aminotransferase (AST), alanine transaminase (ALT)/aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine (CREA) were tested using an automatic biochemical analyzer (Roche COBAS C501). All protocols of animal studies were approved by the Ethical Committee of Zhengzhou University (code number: 2015-0002), and were conducted following the principles outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.11. Statistical Analysis

The experimental data were statistically analyzed according to an independent sample one-way analysis of variance (ANOVA) with the software SPSS 19.0 (SPSS Co., Ltd., Chicago, IL, USA). Through multiple comparisons with Duncan's multiple range test (DMRT), significant differences between the mean values were determined ($p < 0.05$). The results were expressed as the mean ± standard error of three independent experiments.

3. Results

3.1. Isolation and Identification of Antagonistic Strain

From 158 isolated bacteria, seven isolates were able to inhibit *R. cerealis* (Figure 1A). Among them, the highest inhibition rate of strain RB5 on *R. cerealis* reached up to 68.6%. After culturing on the LB plate, the colony of strain RB5 was opaque, smooth, milky white, moist, round, and with neat edges. The strain was an axiolitic, rod-shaped, and Gram-negative bacterium. Moreover, RB5 showed positive results in the nitrate reduction, glucose reaction, citrate, indole, amylolysis, and contact enzyme, whereas it showed negative results for the methyl red and VP experiment. Meanwhile, the 16S rRNA gene sequence of strain RB5 (GenBank accession number: GU232769) was arranged in the phylogenetic tree (Figure 1B). Based on the morphological characteristics, physiological and biochemical analysis, and molecular identification, antagonistic strain RB5 was identified as *P. fluorescens*.

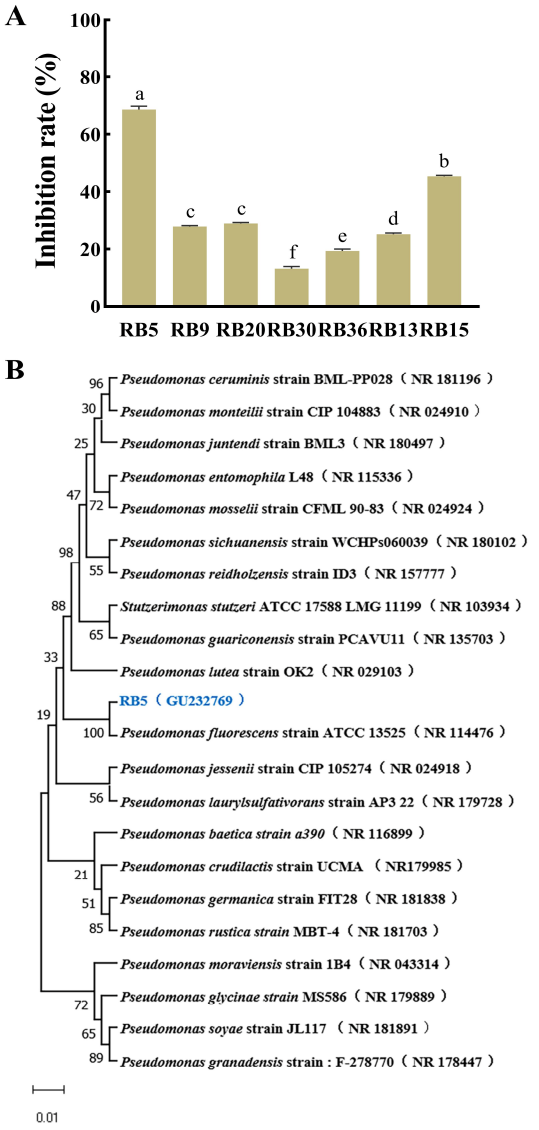

Figure 1. Screening and identification of bacteria antagonistic to *R. cerealis*. (**A**) Inhibition rate of seven strains against *R. cerealis*; (**B**) phylogenetic tree constructed with the 16S rRNA gene sequences. Different lowercase letters on the bars in subfigure A indicate the significant difference at the $p < 0.05$ level using Duncan's multiple range test.

3.2. Hydrolase and Siderophore Produced from Strain RB5

The antifungal and plant growth promoting potential of strain RB5 was evaluated by characterizing several indicators on a plate. The results showed that RB5 had the potential to produce siderophore, protease, and chitinase (Figure 2).

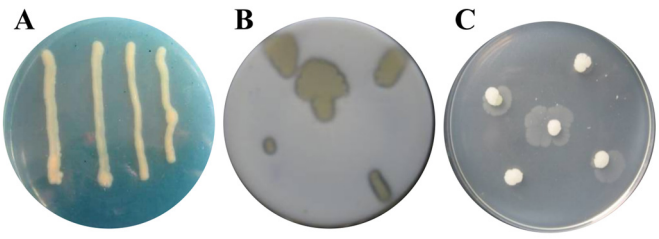

Figure 2. Production of siderophore, protease, and chitinase on the detection plates. (**A**) Siderophores; (**B**) proteases; (**C**) chitinase.

3.3. The Optimal Culture Conditions for RB5 Screened with Response Surface Methodology (RSM)

3.3.1. Single-Factor Optimization

Different nitrogen and carbon sources can affect the inhibition rate of antagonistic strain RB5. As shown in Figure 3, when soybean meal and cassava were taken as a nitrogen source and carbon source, respectively, the inhibition rates of the culture filtrate against *R. cerealis* were the best.

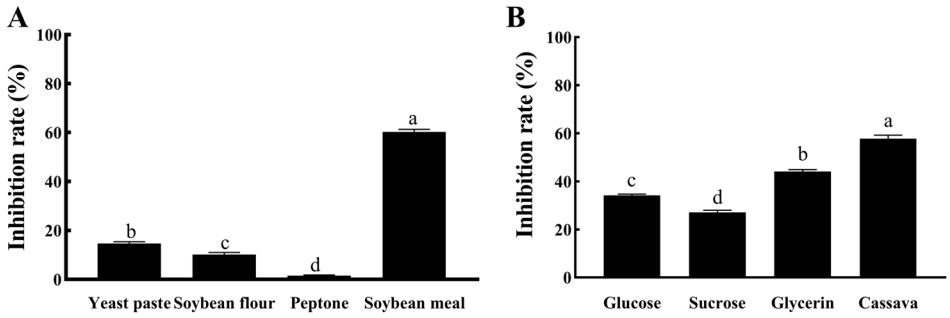

Figure 3. The effects of different nitrogen and carbon sources on the inhibition of the culture filtrate against *R. cerealis*. (**A**) Nitrogen sources; (**B**) carbon sources. Data are the mean ± SE. Different lowercase letters on the bars indicate the significant difference at the $p < 0.05$ level using Duncan's multiple range test.

3.3.2. Selection of the Optimal Variables

According to the results of the single-factor test, the Placket–Burman experiment was conducted with cassava (A), soybean meal (B), temperature (C), pH (D), speed (E), amount of inoculation (F), and culture time (G) as independent variables and inhibition rates as response values. The results are shown in Table 3 and the highest inhibition rate reached up to 73.43%. Among all seven variables, cassava, soybean meal, and speed had great influences on the inhibition rates, which were taken as the main variables.

Table 3. Screening of significant variables using Placket–Burman design.

Numbers	A	B	C	D	E	F	G	Y (Inhibition Rate)/%
1	−1	−1	−1	−1	−1	−1	−1	56.65
2	1	−1	1	1	−1	1	1	61.34
3	1	−1	1	1	1	−1	−1	69.75
4	−1	1	−1	1	1	−1	1	71.29
5	−1	−1	−1	1	−1	1	1	58.47
6	1	1	−1	−1	−1	1	−1	70.13
7	−1	1	1	1	−1	−1	−1	63.51
8	−1	1	1	−1	1	1	1	68.78
9	1	1	−1	1	1	1	−1	73.43
10	1	1	1	−1	−1	−1	1	68.16
11	−1	1	1	−1	1	1	−1	65.67
12	1	−1	−1	−1	1	−1	1	71.35

3.3.3. Determination of Cultivation Conditions with Box–Behnken Design

The Box–Behnken test was conducted with three variables of A, B, and E, and the results are shown in Table 4. When the concentration of cassava is 9 g/L, the concentration of soybean meal is 6 g/L, and the speed is 200 r/min; the highest inhibition rate reached up to 82.37%.

Table 4. Experimental design and results of the Box–Behnken optimization.

Numbers	A	B	E	Y (Inhibition Rate)/%
1	0	0	0	82.37
2	−1	−1	0	68.45
3	1	−1	0	75.51
4	−1	1	0	77.76
5	1	1	0	78.93
6	1	0	1	80.83
7	−1	0	−1	65.38
8	0	1	−1	76.5
9	0	1	1	78.49
10	0	−1	−1	66.87
11	0	0	0	81.65
12	0	−1	1	77.67
13	1	0	−1	76.34
14	0	0	0	81.93
15	−1	0	1	77.52

Through Design Expert software 8.0.6, the response values were analyzed using a regression equation fitted with multiple regression. The regression equation for the inhibition rate is as follows:

$$Y\ (\text{Inhibition rate}) = 81.98 + 2.81 \times A + 2.90 \times B + 3.68 \times C - 1.47 \times A \times B - 1.91 \times A \times C - 2.20 \times B \times C - 3.34 \times A^2 - 3.48 \times B^2 - 3.62 \times C^2$$

The analysis of variance is shown in Table 5. A p value less than 0.05 indicated that the model terms were significant. In this model, the p value was 0.0008, indicating that the model is significant. Meanwhile, model terms A, B, C, A^2, B^2, C^2, AC, and BC are significant.

Table 5. Variance analysis of regression equation.

Type	Sum of Squares	Degree of Freedom	Average of Squares	*F* Value	*p* Value	Significance
Model	397.85	9	44.21	30.21	0.0008	**
A	63.28	1	63.28	43.24	0.0012	**
B	67.16	1	67.16	45.90	0.0011	**
C	108.19	1	108.19	73.94	0.0004	**
AB	8.67	1	8.67	5.93	0.0591	
AC	14.63	1	14.63	10.00	0.0250	*
BC	19.40	1	19.40	13.26	0.0149	*
A^2	41.26	1	41.26	28.20	0.0032	**
B^2	44.66	1	44.66	30.52	0.0027	**
C^2	48.46	1	48.46	33.12	0.0022	**
Residual	7.32	5	1.46			
Lack-of-fit	7.05	3	2.35	17.85	0.0535	
Pure error	0.26	2	0.13			
Total error	405.16	14				

Note: *F* represents Fischer's test; *p* represents probability; '*' indicates a significant effect ($p < 0.05$). '**' indicates a highly significant effect ($p < 0.01$).

According to the regression equation, Design-Expert 7.0 was used to draw the response surface analysis diagram and the contour line. As shown in Figure 4, each response surface represents the interaction between two independent variables. The steeper the trend of the curve, the stronger the interaction and the greater the effect on the inhibition rate. The contour plot visually reflects the significant degree of interaction between the factors. With the increase in any two variables, the inhibition rate tended to increase. After the peak of the interaction, the surface decreased. The response surface lines corresponding to the

three factors are similar, indicating that all have a significant effect on the inhibition rate. According to the analysis of RSM, the optimal culture conditions are 8.7 g/L of cassava, 5.2 g/L of soybean meal, pH 6.8, a 218 r/min speed, a 31.5 °C temperature, and 54 h of culture time. The inhibitory rate of the antagonistic bacterium RB5 reached 87.012%.

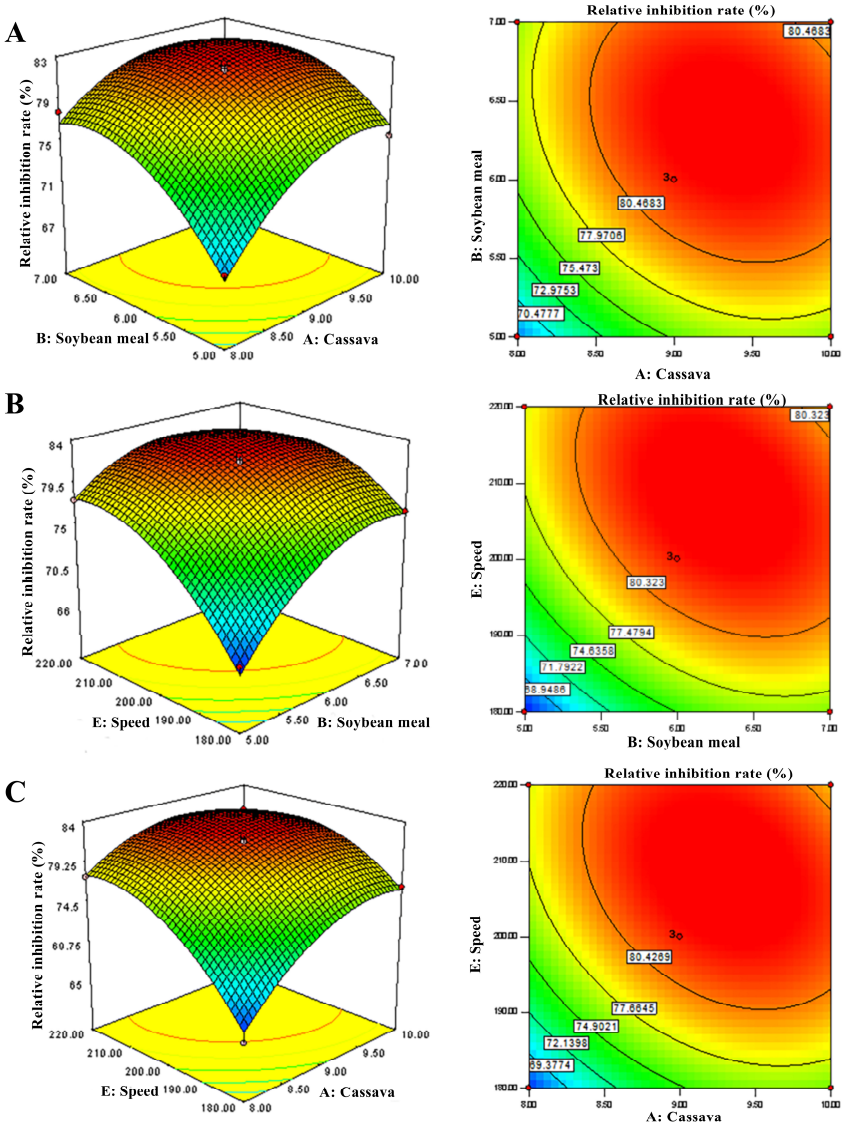

Figure 4. Response surface analysis diagram and contour lines of the interaction between the concentration of cassava and soybean meal and rotation speed. (**A**) Cassava and soybean meal; (**B**) speed and soybean meal; (**C**) cassava and speed.

To test the reliability of the results obtained with RSM, the optimal culture conditions were adopted for culture strain RB5. The inhibition rate of the RB5 culture filtrate was 79.06%, and the relative error is 8% less than the theoretical value. Therefore, the culture conditions of strain RB5 optimized based on RSM are accurate, feasible, and with practical value.

3.4. Antifungal Activity of RB5 Culture Filtrate

To evaluate the antifungal activity of the culture filtrate, we detected the effect of different concentrations of the culture filtrate on *R. cerealis*. As shown in Figure 5A,B, the mycelial diameter of *R. cerealis* was significantly decreased with an increase in the concentrations of the culture filtrate on the PDA plate, resulting in a gradual increase in the inhibition ratio of the culture filtrate against *R. cerealis.*

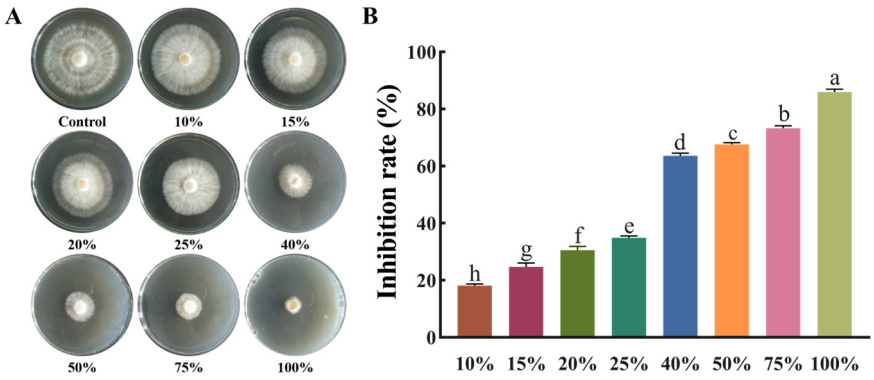

Figure 5. Inhibition of the RB5 culture filtrate on *R. cerealis*. (**A**) The culture filtrate with different concentrations showed inhibition activity; (**B**) the inhibition rates of different concentrations of the culture filtrate. Data are the mean ± SE. Different lowercase letters on the bars indicate the significant difference at the $p < 0.05$ level using Duncan's multiple range test.

3.5. Effect of RB5 Culture Filtrate on Mycelial Morphology of R. cerealis

The changes in the mycelial morphology of *R. cerealis* were observed under the optical microscope (Figure 6) and showed that mycelia involved turgidity and shrinkage in the RB5 culture filtrate treatment group, particularly at the mycelia tips. This suggests that the culture filtrate can remarkably affect the mycelial morphology of *R. Cerealis*.

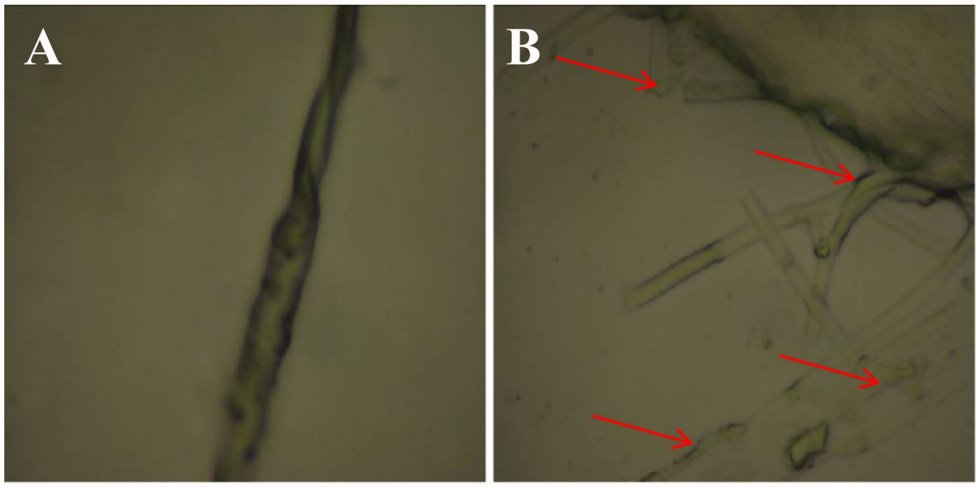

Figure 6. Inhibition of the RB5 culture filtrate on the mycelial morphology of *R. cerealis*. (**A**) Control; (**B**) the culture filtrate of RB5. Red arrows indicate the turgidity or shrinkage of mycelia.

3.6. Effects of RB5 Culture Filtrate on Cell-Wall-Degrading Enzymes of R. cerealis

Most plant pathogenic fungi produce cell-wall-degrading enzymes (CWDEs) to degrade cell walls of a host plant for invasion. As shown in Table 6, enzyme activities of PG, PMG, and PMTE of *R. cerealis* were decreased after treating with a different concentration of the RB5 culture filtrate (40%, 50%, 75%, and 100%). As the concentration of the culture filtrate increased, the activities of the three enzymes significantly decreased. This result indicates that RB5 controls disease by inhibiting the cell-wall-degrading enzymes of pathogenic fungi.

Table 6. Activities of cell-wall-degrading enzymes of *R. cerealis* treated with different concentrations of RB5 culture filtrate.

Culture Filtrate (%)	PG (U/mg)	PMG (U/mg)	PMTE (U/mg × 10^{-3})
0	12.00 ± 0.58 ^{a}	43.00 ± 2.08 ^{a}	14.29 ± 2.31 ^{a}
40	5.53 ± 0.14 ^{b}	15.44 ± 0.17 ^{b}	5.93 ± 0.63 ^{b}
50	3.40 ± 0.10 ^{c}	4.60 ± 0.32 ^{c}	2.07 ± 0.30 ^{c}
75	2.21 ± 0.14 ^{d}	1.71 ± 0.03 ^{cd}	0.87 ± 0.12 ^{c}
100	1.46 ± 0.70 ^{d}	0.87 ± 0.21 ^{d}	0.76 ± 0.66 ^{c}

Note: Data are the mean ± SE. Different lowercase letters indicate the significant difference at the $p < 0.05$ level using Duncan's multiple range test.

3.7. Biocontrol Effects of RB5 Culture Filtrate on Wheat Sheath Blight

The biocontrol efficacy of strain RB5 against wheat sheath blight was evaluated using wheat seedlings in pots. The disease incidence rate, disease index, and control efficacy are presented in Table 7. The results showed that the control efficacies of strain RB5 became higher along with the concentration of the RB5 suspension increasing. After the RB5 suspension (1×10^9 cfu/mL) treatment, the disease index of wheat seedlings was 38.57% and the control efficacies of strain RB5 were 71.22%. However, the control efficacies of *carbendazim* were 70.69%. Therefore, compared to chemical pesticides, the control effect of RB5 was higher, which indicates that the RB5 culture filtrate has great application value.

Table 7. Biocontrol efficacy of RB5 on wheat sheath blight.

Treatments	Disease Incidence Rate (%)	Disease Index	Control Efficacy (%)
Sterile water	97.40 ± 1.67 ^{a}	54.15 ± 1.23 ^{a}	--
Carbendazim	34.12 ± 1.86 ^{d}	16.26 ± 1.51 ^{d}	70.69 ± 2.17 ^{a}
RB5 (1×10^5 cfu/mL)	48.23 ± 2.23 ^{b}	33.75 ± 1.65 ^{b}	37.67 ± 3.51 ^{c}
RB5 (1×10^7 cfu/mL)	40.78 ± 1.58 ^{c}	20.91 ± 1.78 ^{c}	61.38 ± 3.32 ^{b}
RB5 (1×10^9 cfu/mL)	32.23 ± 3.24 ^{d}	15.58 ± 1.34 ^{d}	71.22 ± 1.78 ^{a}

Note: Data are the mean ± SE. Different lowercase letters indicate the significant difference at the $p < 0.05$ level using Duncan's multiple range test.

3.8. Safety Evaluation of RB5 Culture Filtrate on Mice

3.8.1. Effects of RB5 Culture Filtrate on Weight and Growth of Mice

After 7 days of the oral administration of the RB5 culture filtrate to mice, there was no significant difference in body weights between the control group and treatment group. At the same time, there were no deaths, and the mice appeared to be healthy without showing significant behavioral or morphological abnormalities (Table 8).

Table 8. Effects of culture filtrate on the body weight, behavior, and mortality rate of mice.

Items	Body Weight		Behavior	Mortality Rate (%)
	Initial Weight (g)	Final Weight (g)		
Control ♂	20.89 ± 1.72 ^{a}	46.32 ± 1.25 ^{a}	Normal	0
Control ♀	20.36 ± 1.68 ^{a}	44.64 ± 1.62 ^{a}	Normal	0
Treatment ♂	20.68 ± 1.74 ^{a}	45.16 ± 1.46 ^{a}	Normal	0
Treatment ♀	20.12 ± 1.64 ^{a}	44.57 ± 1.78 ^{a}	Normal	0

Note: Data are the mean ± SE. Different lowercase letters indicate the significant difference at the $p < 0.05$ level using Duncan's multiple range test.

3.8.2. Effects of RB5 Culture Filtrate on Serological Physiological Indexes of Mice

The effects of the RB5 culture filtrate on serological physiological indexes are presented in Table 9. There was no significant difference of serological physiological indexes between

the control group and the treatment group. Thus, the results indicate that the RB5 culture filtrate is not harmful to mice.

Table 9. Effects of RB5 culture filtrate on the serological physiological indexes of mice.

Items	Serological Physiological Index				
	ALT ($U \cdot L^{-1}$)	AST ($U \cdot L^{-1}$)	ALT/AST ($U \cdot L^{-1}$)	BUN ($mmoL \cdot L^{-1}$)	CREA ($\mu mol \cdot L^{-1}$)
Control ♂	57.67 ± 4.50 ^{a}	120.33 ± 5.51 ^{a}	2.60 ± 0.12 ^{a}	12.45 ± 0.79 ^{a}	24.89 ± 3.96 ^{a}
Treatment ♂	59.66 ± 5.12 ^{a}	121.24 ± 5.69 ^{a}	2.71 ± 0.17 ^{a}	12.40 ± 0.88 ^{a}	24.17 ± 2.17 ^{a}
Control ♀	46.33 ± 1.52 ^{b}	108.67 ± 7.64 ^{a}	1.89 ± 0.13 ^{b}	12.39 ± 0.82 ^{a}	19.20 ± 2.41 ^{b}
Treatment ♀	44.67 ± 1.53 ^{b}	109.56 ± 6.51 ^{a}	1.85 ± 0.27 ^{b}	11.02 ± 0.37 ^{a}	19.10 ± 0.67 ^{b}

Note: Data are the mean ± SE. Different lowercase letters indicate the significant difference at the $p < 0.05$ level using Duncan's multiple range test. ALT represents alanine transaminase, AST represents aspartate aminotransferase, ALT/AST represents alanine transaminase/aspartate aminotransferase, BUN represents blood urea nitrogen, CREA represents creatinine.

4. Discussion

Wheat sheath blight is a serious disease of wheat in China. As the pathogenic fungus of wheat, *R. cereasis* is soil-borne, and once conditions are favorable, it will rapidly develop and infect plants [41]. Developing improved microorganisms is the most important and environmentally friendly way to control this plant disease. In the current study, strain RB5 from soil was identified as *P. fluorescens* and had the best inhibition rate activity against *R. cereasis among isolated strains*. Meanwhile, some reports have showed the antagonistic microorganisms could produce extracellular β-N-acetyl hexosaminidase, chitin 1,4-β-chitotriosidase, glucan 1,3-β-glucosidase, and could have protease, which can substantially destroy the cell wall structure and hence retards fungal mycelium growth [5]. Zhou et al. [42] reported that *Bacillus cereus* YN917 could produce protease, amylase, cellulase, IAA, ACC deaminase, and siderophores, and has the activity of decomposing mineral phosphate. It has been proved that *Bacillus velezensis* NKG-2 produces cellulase, chitinase, β-glucanase, IAA, and siderophores [43]. Different genera have a different enzyme production capacity [18]. In this study, *P. fluorescens* RB5 could produce proteases, chitinase, and siderophores. A siderophore chelates with ions in the rhizosphere, preventing the pathogen from receiving sufficient iron nutrition, and reducing the harm of the pathogen to plants, thereby improving plant growth and development [44].

Response surface methodology (RSM) is a method of simulating the absolute limit-state surfaces through a series of multivariate deterministic tests. For example, Ran et al. [45] improved the antagonism of *Paenibacillus polymyxa* against *Fusarium graminearum* through RSM. In the present study, to optimize the culture conditions of strain RB5, different cultivation conditions (nutrient sources, pH, temperature, and rotary speed) were analyzed with RSM. During the test trials, to reduce manufacturing costs and facilitate industrialization, cassava was selected as a carbon source and soybean meal as a nitrogen source for further optimization. Currently, the utilization of these ingredients has become prevalent in industrial fermentation processes due to their widespread availability and applicability to nutrient requirements in various fermentation applications [46]. Finally, under the optimal cultivation condition, the inhibition rate of the RB5 culture filtrate on *R. cerealis* reached 79.06%. This result indicates that RSM was an efficient method to optimize the bacterial culture condition.

In order to reveal the mechanism of *P. fluorescens* RB5 inhibiting pathogens, we found that the RB5 culture filtrate would lead to the abnormal growth, turgidity, and shrinkage of mycelia through optical microscope observation. Saoussen Ben Khedher et al. [47] also pointed out that *Bacillus subtilis* V26 caused the hyphae of *Botrytis cinerea* to swell and deform. Cell-wall-degrading enzymes (CWDEs) produced from pathogenic fungi play a role in the invasion of plants by pathogens [48,49]. They can contribute to pathogenesis by degrading the wax, cuticle, and cell walls of host plants, thus aiding tissue invasion

and pathogen dissemination [48]. Therefore, it is important to control plant diseases by decreasing the activities of CWDEs from pathogens. In this study, we investigated the activity changes of CWDEs of *R. cerealis* after treating with RB5 culture filtrates. The result indicates that the culture filtrates reduce the CWDEs' activities, which is in accordance with the report of Ranjbar et al. that thymol against the main fungi inhibit pomegranate fruit rot by suppressing the production of cell-wall-degrading enzymes [50].

Pseudomonas spp. is beneficial for a soil environment to promote root growth [51]. It has the advantages of a fast reproduction, a strong adaptability, an easy artificial cultivation, a stable preparation, an easy application, no pollution of the environment, and the control of a variety of plant diseases [52]. The biocontrol effect of strain RB5 to control *R. cerealis* was confirmed with the efficacy of 71.22%. However, during the use of microbial inoculants, the safety of strains is very important for their application. It is necessary to evaluate the safety of a bacterium before its application. Acute toxicity tests on mice were conducted to evaluate the safety of RB5. Mice were chosen because they are considered as the most sensitive laboratory animals [53]. Moreover, serology is a crucial index of safety evaluation. In the present study, various indexes of mice have no differences after the administration of the RB5 culture filtrate, indicating that strain RB5 has no adverse effect on the health of mice and can be considered safe for application in agriculture. The result is consistent with the report that the culture filtrate of *B. mojaves* B1302 is safe and can be prepared for microbial fungicides [18]. This study provides a good biocontrol strain for controlling wheat sheath blight.

5. Conclusions

P. fluorescens RB5 showed antagonism against *R. cerealis.* Through optimizing with response surface methodology, the optimal culture conditions of strain RB5 are 8.7 g/L of cassava, 5.2 g/L of soybean meal, pH 6.8, a 218 r/min speed, a 31.5 °C temperature, and 54 h of culture time. The culture filtrate of *P. fluorescens* RB5 had a good inhibitory effect on the mycelial growth, caused the swelling and rupture of the mycelium of *R. cerealis*, and inhibited the activities of three cell-wall-degrading enzymes of *R. cerealis.* The RB5 suspension involved an effective control for wheat sheath blight with an efficacy of 71.22%. Under the treatment of the culture filtrate, the disease incidence and index of wheat seedlings were consistent with those of commercial pesticides. Furthermore, the RB5 culture filtrate is safe to use and could be developed into an ecological-friendly biological control agent for wheat sheath blight.

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