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Biocontrol Potential of Antagonistic Bacteria against *Sclerotium rolfsii* and *Fusarium graminearum*: Isolation, Identification, and Mode of Action

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Abstract

Given the escalating threats of soil-borne diseases to global agriculture, this study investigates the biocontrol potential of *Bacillus* species against peanut southern blight (caused by *Sclerotium rolfsii*) and wheat crown rot (caused by *Fusarium pseudograminearum*). Strains of Soil 1-2, Soil 1-3, Soil 1-59, and Soil 2-93 were isolated from wheat rhizosphere soil and demonstrated strong antagonistic activity against both pathogens, with inhibition rates exceeding 53% in dual-culture assays. Based on morphology, biochemical characteristics, and molecular evolution analyses, the strains were identified as *B. velezensis*, *B. subtilis*, *B. atrophaeus* and *B. pumilus*, respectively. Further experiments revealed that cell-free filtrates of these strains exhibited significant antifungal activity, particularly against *S. rolfsii*, with complete inhibition at 72 hours. The strains also showed broad-spectrum antagonism against other fungal pathogens. Pot trials indicated that cell-free filtrates of Soil 1-2 and Soil 1-59 significantly reduced peanut southern blight severity and enhanced plant fresh weight and defense-related enzyme activities. Antifungal mechanisms included the production of volatile organic compounds, extracellular enzymes (protease, cellulase, amylase), and crude proteins. This study confirms the potential of specific *Bacillus* strains as effective biocontrol agents and highlights their multifaceted modes of action, providing a foundation for future development of sustainable disease management strategies.

Keyword: Biocontrol bacteria, Identification, Inhibition mechanism, Soil-borne diseases.

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1. Introduction

Soil-borne diseases represent a major threat to global agricultural productivity, causing substantial economic losses and impeding the development of sustainable, high-efficiency agriculture. **Wheat is a fundamental cereal crop and play a major role in China's food security. Peanut is a vital oil and economic crop, and it is also a major rotational crop grown after wheat. Wheat crown rot and peanut southern blight are two important soil-borne diseases. *F. pseudograminearum* is the primary causal agent of wheat crown rot and could substantially reduce wheat production (Zhou et al., 2019). It is also a global plant disease and** prevalent in many countries and regions (Smiley et al., 2005). **While peanut southern blight is** caused by *S. rolfsii* and poses a significant threat to peanut production, leading to yield losses ranging from 10% to 80%, and in severe cases, can result in complete crop failure (Kunrong et al., 2018).

Chemical pesticides are an important means of controlling plant diseases. But chemical pesticides accelerate the development of pathogen resistance and raises serious concerns regarding environmental pollution, pesticide residues, and food safety. In addition, soil physicochemical properties can also limit the effectiveness of chemical pesticides against soil-borne diseases. Microbial-based disease control is a viable, environmentally friendly strategy with a low risk of resistance development (Xu et al., 2023). Due to its multiple biocontrol mechanism, including extracellular enzymes, volatile gases, antimicrobial compounds, and the induction of systemic plant defenses, it is recognized as a promising method to control soil-borne diseases. **However, most studies have focused on only a single crop disease in pot experiments. This leads to the application of different biocontrol agents into the soil for the control of different diseases. Such practice not only continuously subjects the soil microbial community to severe disturbance by exogenous agents, but also increases the cost for farmers. So, it is necessary for farmers to develop broad-spectrum biocontrol strategies to control multiple soil-borne pathogens.**

Therefore, we attempted to isolate and screen for microorganisms with antagonistic

activity against these two pathogens from wheat rhizosphere soil. Subsequently, pot experiments were conducted to determine the antimicrobial activity of their fermentation broth and the induced plant defense responses. Finally, preliminary investigations into the inhibition mechanisms were carried out by analyzing the inhibitory activities of crude proteins and volatile gases of the microorganisms.

2. Materials and methods

2.1. Bacteria isolation, screening, and identification

Bacterial strains were isolated from wheat rhizosphere soil. The roots were placed in 10 ml of sterile water and shake at 180 rpm for 15 min. The prepared soil suspension was diluted by a factor of 10^{-7}, and 200 µL dilution was coated on LB plates and cultured at 28 ℃ for 2-5 days. Single colonies with unique morphology were selected for antagonist activity screening through plate confrontation.

The pathogens of *S. rolfsii* (Zhi et al., 2024) and *F. pseudograminearum* (Zhou et al., 2019) obtained from our laboratory were used to screen the antagonist bacteria. A 6 mm diameter of each mycelium plug was placed in the center of PDA plate and the isolated bacteria was streak-inoculated at positions 0.5 cm away from the plug. The control treatment only inoculated mycelium plug. Each treatment was triplicate and cultured at 28 ℃ for 3-5 days. The inhibition rate was calculated as follows: **Inhibition rate (%) = (control group mycelium diameter - treatment group mycelium diameter) / (control group mycelium diameter - 6) × 100% (Zhi et al., 2024).**

Morphological, biochemical, and molecular analyses were carried out to identify the strains. LB plate was used to observe the color, surface, and shape of the colony. Biochemical analysis was carried on according to the methods in Bergey's Manual of Systematic Bacteriology (Garrity, 2001). Various carbon sources have been used to assess the catalase activity of the strains. Oxidase test, catalase test, indole product test, nitrate reduction, citrate utilization, malonate utilization, urease product detection, gelatin liquefaction, starch hydrolysis, the methyl red test, and the Voges Proskauer

test were conducted to determine the strain's biochemical profile. The experiments were conducted three times with three replicates under the same conditions.

DNA of these bacteria were extracted by the DNA extraction kit (Solarbio, Beijing, China). Phylogenetic tree based on *16S rRNA* and *gyrB* gene sequences was used to identify the strains. The *16S rRNA* gene was amplified using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5 '- GGTTACCTTGTTACGATT-3'). PCR amplification program: 98℃ for 2 min; 35 cycles at 98℃ for 30 seconds, 55℃ for 10 seconds, and 72℃ for 20 seconds; 72℃ for 2 min; and a final extension at 72°C for10 min. *GyrB* gene was amplified using primers UP1F (5'-GAAGGTCATGACCGTTCTGCA-3') and UP2R (5'-AGAGAGGGTACGGATGGTGCGAGCC-3'). PCR amplification program: 94℃ for 4 min; 35 cycles at 94℃ for 1 min, 57℃ for 10 seconds, and 72℃ for 20 seconds; 72℃ for 1 min; and a final extension at 72°C for10 min. The PCR products were sequenced by **Sangon Biotechnology Co., Ltd.** (Shanghai, China), and the obtained sequences were subjected to BLAST similarity analysis on NCBI. The phylogenetic tree was constructed by MEGA-X software.

2.2 Antifungal effect of cell-free filtrate

Bacteria cells were harvested by scraping the lawn from the 24-h-old plate, inoculated into 50 mL of LB liquid medium, and incubated at 28 °C with shaking at 180 r/min for 12 h to prepare the seed culture. Transfer the seed cultures into fresh liquid LB medium at a 2% inoculum size (in 100/250 mL flasks) and shake at the same condition for 72 h. After that, sampling was initiated at the beginning and performed at 4-h intervals, and the optical density at 600 nm (OD_{600}) was determined with a spectrophotometer. The growth curve was constructed by plotting OD values (or log CFU/mL) against incubation time (h).

A longer fermentation period is associated with increased production of bioactivated secondary metabolites. Based on preliminary experiments, the fermentation broth harvested at 48 h and that at 72 h were used for experiments against *S. rolfsii* and *F.*

pseudograminearum, respectively. After that, the supernatant was collected and filtered through a 0.22-μm sterile membrane to obtain a cell-free filtrate. Cell-free filtrate medium was prepared by PDA medium and cell-free filtrate with the ratio at 1:1 (v/v). After that, a 6 mm diameter of *S. rolfsii* and *F. pseudograminearum* fungal mycelium plugs were inoculated onto the center of the mixed medium. The control plate was the mixture of liquid LB medium and PDA medium. The inhibition rate was calculated when the mycelium covered approximately 2/3 of plate area.

2.3 Antifungal spectrum of these bacteria

Plate confrontation assay also been used to assess the antifungal activity of the screened strains to other fungal pathogens. **Pathogenic agents as** *S. rolfsii*, *F. graminearum*, *F. pseudograminearum*, *F. oxysporum*, *Ceratobasidium graminearum*, *Phytophthora capsici*, *Colletotrichum gloeosporioides*, *Botryosphaeria dothidea*, and *Corynespora cassiicola* have been used to evaluate the antifungal spectrum of the **selected** bacteria. PDA plate also was selected in this part and each treatment was replicated three times. In addition, the antifungal spectrum of the cell-free filtrates also has been analyzed based on above method.

2.4 Pot experiment of cell-free filtrate

2.4.1 Peanut

Uniformly sized Yuhua 22 peanut seeds were disinfected with 75% ethanol, rinsed by sterile water, and subjected to germination promotion at 30 °C for 3 d. Two germinated seeds were sowed in a plastic pot (7 cm×7 cm×8 cm) and maintained at 30 ℃ and 80% relative humidity. After 7 d, the uniformed seedlings were selected for subsequent experiments.

First, 6-mm plugs were punched from plates covered with S. rolfsii mycelia. The plugs were then transferred with forceps and placed mycelial-side in close contact with the stem base of peanut. 7 days after inoculation, 10 mL 48h cell-free filtrate was irrigated to peanut roots. The control treatments were irrigated with liquid

LB medium. All treatments were repeated 3 times. The incidence was investigated at 7 d after inoculation. The disease severity of peanut was according to Dong et al. (Dong et al., 2001). Following formulas were used to calculate disease index and control efficacy.

Disease index (%) = [Σ (amount of the diseased plant × corresponding disease scale) / (amount of the tested plant × 9)] × 100%.

Control efficacy (%) = [(the disease index of control - the disease index of treatment) / the disease index of control] ×100%.

2.4.2 Wheat

The Aikang 58 wheat seeds were sterilized by 75% alcohol, and then pregermination at 28°C for 24 h. **In each small pot, 8 wheat seeds with consistent germination status were sowed and then covered with soil.**

One day after sowing, 1.5 g *F. pseudograminearum* millet grain inoculum was lay on the base of the wheat stem, and covered the inoculum with soil. When the pathogen invaded, 10 mL 72 h cell-free filtrate was irrigated to wheat roots. The control treatment was the seedlings with LB medium. **After 21 days inoculation, investigated the disease incidence of wheat.** *F. pseudograminearum* millet grain inoculum, **disease severity, and rating scales were all according to Zhou *et al.*** (2019). **The disease index and control efficacy were calculated based on the formulas in 2.4.1.**

2.5 Effect of cell-free filtrate on peanut growth-promoting and defense activity

Peanut seeds were disinfected. After 7 d of the seed germination in pots, the seedlings were irrigated with 2 mL filtered broth each two days. Peanut plants were uprooted after 14 days. The roots were cleaned by water and blotted dry. And then, shoot fresh weight, shoot dry weight, plant height, root length, and underground dry weight were measured. **The enzyme activities of catalase (CAT), phenylalanine ammonia-lyase (PAL), and polyphenol oxidase (PPO) were determined by spectrophotometry**

according to Suzhou Keming Biotechnology Co., Ltd. reagent kit protocols.

2.6 Mechanism of antifungal action

2.6.1 Antifungal effect of bacterial volatile

Petri dish mouth-to-mouth fumigation method has been used to evaluate the antifungal effect of bacterial volatile gases on pathogenic fungi (He et al., 2020). The antagonistic strains cultured for 24 h were densely inoculated to LB plates, and the *S. rolfsii* plug was inoculated in the center of the PDA plate. Putted these two plates mouth-to-mouth and sealed the seam. The plate with *S. rolfsii* plug was on the top of bacteria. The mouth-to-mouth plates without bacteria was the control. The inhibition rate calculation was the same as 2.4.

2.6.2 Antifungal effect of crude enzymes

Bacterial enzymes are also the effective tool to inhibit pathogenic fungi. Ammonium sulfate precipitation and dialysis have been normally used to prepare the crude enzymes (Jana et al., 2025). The cell-free filtrate was obtained according to 2.2. Firstly, the cell-free filtrates were kept in an ice bath, and the ammonium sulfate was gradually added until 60% saturation was reached. During this process, the mixture was stirred constantly. After the ammonium sulfate dissolved, the mixture was kept in 4 ℃ freezer overnight. 15 KDa dialysis bag has been used to remove ammonium sulfate and collect proteins. The mixture was centrifuged and collected flocculent precipitate. The flocculent precipitate was redissolved and placed in dialysis bag. And then, putted the bag in PBS buffer (pH=7.4) at 4 ℃ to desalt ammonium sulfate. The dialysis buffer was replaced each 4 hours. After dialysis, the sample was lyophilized and stored at -20 °C. Dry protein powder was dissolved in sterile water and kept the concentration at 10 mg/L. The solution mixed with PDA resulting in a final concentration of 5 mg/mL for crude protein. And then, prepared culture medium plates. PBS buffer mixed wiht PDA as a control. *S. rolfsii* plug was inoculated in the center of the PDA plate at 28℃

for 2 days. The inhibition rate was calculated as mentioned above.

2.6.3 Extracellular enzyme activity test

In order to better understand the biocontrol mechanism of these strains, the strains were separately inoculated on skim milk agar plate (Kazanas, 1968), cellulose agar plate (Teather and Wood, 1982), and starch-containing agar plate (Kammoun et al., 2008) to analyze the activities of proteinase, cellulase, and amylase. The formula of protease identification medium was fresh skim milk 100 mL/L, yeast extract 5.0 g/L, NaCl 5.0 g/L, agar 20 g/L, adjusted to 1 L with deionized water, pH 7.2±0.1 (25°C). The formula of diastase identification medium was soluble starch 10 g/L, peptone 5 g/L, NaCl 0.5 g/L, agar 20 g/L, pH 7.2±0.1 (25°C). The formula of cellulase identification medium was carboxymethyl cellulose 2 g/L, peptone 5 g/L, NaCl 0.5 g/L, agar 20 g/L, pH 7.2±0.1 (25°C). **The strains were separately inoculated in the testing medium, and kept in 28°C for 3-5 days. After cultivation, the presence of a clear zone around the bacteria colony suggested that the bacteria have the enzyme activity.**

2.7 Statistical analysis

All experimental data were expressed as mean ± standard deviation (SD). Comparisons among multiple groups were conducted using one-way ANOVA. When a statistically significant difference was detected, post hoc multiple comparisons were further performed using Tukey's honestly significant difference test.

3. Results

3.1 Bacteria isolation and screening

There were 265 bacteria isolated from wheat rhizosphere soil. All of them have been used to initially screen out antagonistic strains. The strains of Soil 1-2, Soil 1-3, Soil 1-59, and Soil 2-93 showed good inhibitory effects on the growth of *S. rolfsii* and *F. pseudograminearum* with inhibition rates all exceeding 53% (Table 1 and Figure 1).

Table 1. Inhibition rates (%) of four bacteria.

	Soil1-2	Soil1-3	Soil1-59	Soil2-93
S. rolfsii	81.76±0.00a	76.79±2.34ab	67.70±1.47c	71.50±4.74bc
F. pseudograminearum	87.17±0.72a	80.51±2.90a	68.45±3.36b	52.66±2.71c
F. graminearum	69.51±0.86a	65.85±0.86a	58.67±2.16b	-
P. capsici	67.41±4.20a	55.61±0.79a	33.33±8.57b	52.97±5.52a
C. graminearum	83.42±1.35a	83.97±0.78a	65.19±1.35b	85.39±0.79a
C. gloeosporioides	89.88±2.22a	82.78±0.84b	64.28±1.45c	39.35±2.41d
F. oxysporum	70.76±2.51a	59.48±1.91b	52.22±3.14d	-
B. dothidea	64.11±1.17ab	58.37±1.17b	69.78±5.6a	64.83±2.05ab
C. cassiicola	67.97±1.37ab	71.34±2.75ab	74.86±4.74a	64.24±2.09b

Note: Different lowercase letters indicate statistically significant differences.

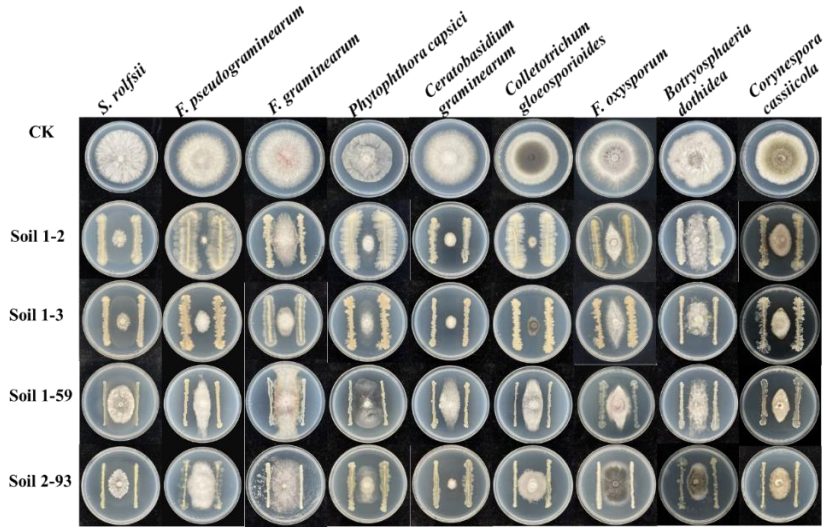

Figure 1. Plate confrontation results of four bacteria against nine pathogens.

3.2 Bacteria identification

The colony of Soil 1-2 were creamy-white with smooth margins; after 5 days, the colony centers transitioned from convex to depressed with noticeable wrinkling. The colonies of Soil 1-3 and Soil 1-59 were creamy-white, circle, dry, and non-transparent, and then turned to light yellow. For Soil 2-93, the colony was white, smooth, and moist. All of them were gram-positive bacteria based on Gram staining (Figure 2).

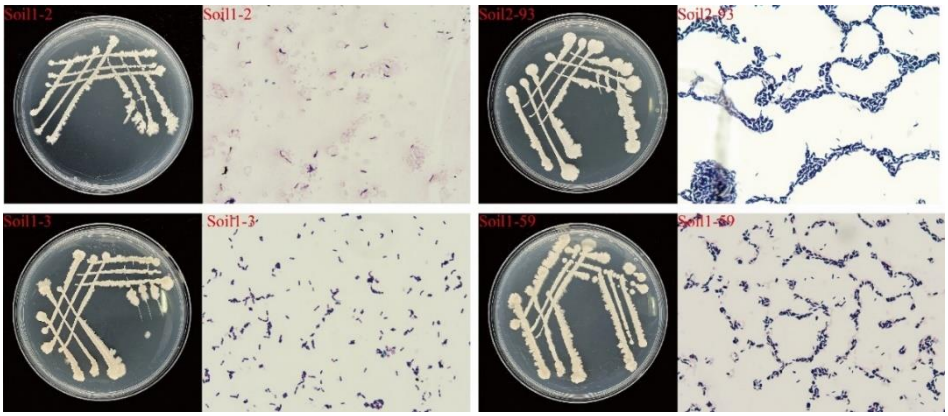

Figure 2. Morphology and gram staining of four bacteria.

Biochemical experiments (Table 2) indicated that these strains showed positive reaction on V-P reaction, catalase assay, starch hydrolysis, gelatin liquefaction, milk peptonization, indole production, D-glucose, L-arabinose, D-xylose, and D-mannitol. Soil 1-2, Soil 1-3, and Soil 1-59 reduced nitrate, and they were negative on MR tests. Based on these results and Bergey's Manual of Determinative Bacteriology, these strains were identified as *Bacillus* genus.

Table 2. Physiological and biochemical characteristics of these four bacteria.

	Soil 1-2	Soil 1-3	Soil 1-59	Soil 2-93
catalase	+	+	+	+
VP	+	+	+	+
MR	-	-	-	-
Starch hydrolysis	+	+	+	-
Nitrate reduction	+	+	+	-
gelatin liquefaction	+	+	+	+
milk peptonization	+	+	+	+
H_2S production	+	+	+	+
Indole production	+	+	+	+
D-glucose	+	+	+	+
L-arabinose	+	+	+	+
D-xylose	+	+	+	+
D-mannitol	+	+	+	+

16S *rRNA* and *gyrB* genes sequences of these strains were 1500-1200 bp in length. The DNA sequences have been submitted to China National Center for Bioinformation, and the access number is PRJCA049187. These two gene sequences of all the strains were highly conserved. Therefore, the combined sequences of 16S *rRNA* and *gyrB* were used to construct a phylogenetic tree (Figure 3). The strains clustered in distinct branches: Soil 1-2 grouped with *B. velezensis* (99% similarity), Soil 1-3 grouped with *B. subtilis* (100%), Soil 1-59 grouped with *B. atrophaeus* (99%),

and Soil 2-93 grouped with *B. pumilus* (100%).

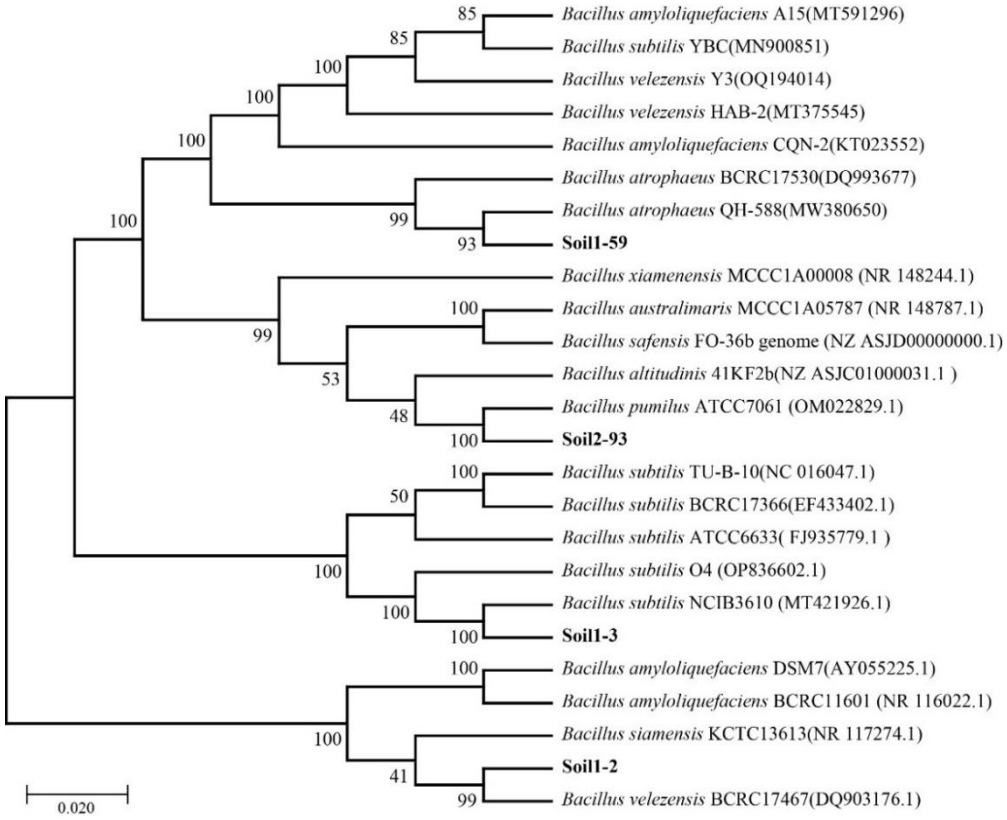

Figure 3. Phylogenetic evolution tree based on 16S rRNA and *gyrB* gene sequence.

3.3 Antifungal spectrum of these bacteria

Soil 1-2 and Soil 1-3 also inhibited other 7 pathogens with inhibition rates > 50%. Soil 1-59 has little inhibitory effect on *P. capsica* growth, and Soil 2-93 has no effect on *F. graminearum* and *F. oxysporum* (Figure 1 and Table 1).

3.4 Bacteria growth curve

These four strains grew rapidly, with cell numbers increasing sharply during the first 12 h, corresponding to the exponential growth phase (Figure 4). After 12 h, the growth rate stabilized, and the cultures essentially entered the stationary phase.

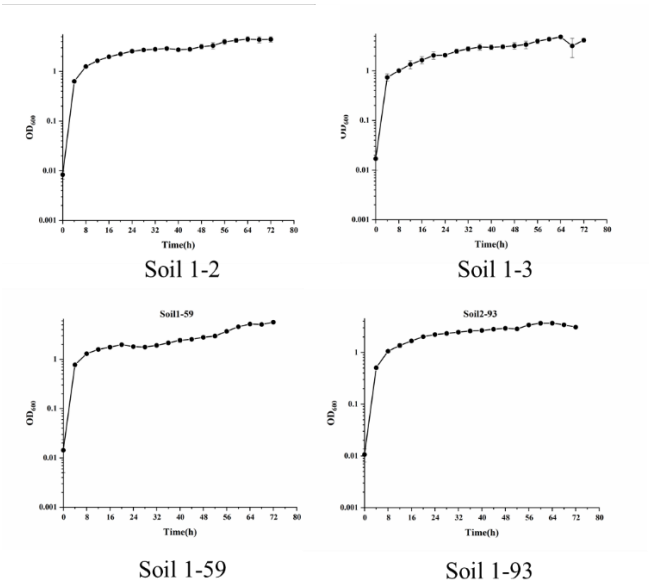

Figure 4. Growth curves of strain Soil 1-2, Soil 1-3, Soil 1-59, and Soil 2-93.

3.5 Antifungal effect of cell-free filtrate

The cell-free filtrates of these four bacteria completely inhibited *S. rolfsii*. For *F. pseudograminearum*, inhibition rates of Soil 1-2 and Soil 1-3 were > 90%; but, the inhibition rates of Soil 1-59 and Soil 2-93 were lower than 51% (Table 3).

The inhibition rates Soil 1-2 and Soil 1-3 cell-free filtrates for *F. oxysporum*, *P. capsici*, *C. graminearum*, *B. dothidea*, and *C. cassiicola* were > 62%. In addition, the cell-free filtrates of Soil 1-59 and Soil 2-93 also significantly inhibited *C. graminearum*, *B. dothidea*, and *C. cassiicola* (Table 3).

Table 3. Inhibition rates (%) of cell-free filtrate on pathogens.

	Soil 1-2	Soil 1-3	Soil 1-59	Soil 2-93
S. rolfsii	100±0.00a	100±0.00a	100±0.00a	100±0.00a
F. pseudograminearum	95.91±0.07a	92.92±0.25a	50.79±0.27b	42.23±0.19c
F. graminearum	66.67±0.53b	83.72±0.37a	40.93±0.06d	59.53±0.00c
P. capsici	100±0.00a	71.90±1.33a	18.58±0.21b	28.10±0.91b
C. graminearum	62.60±0.12b	100±0.00a	83.33±0.85a	100±0.00a
C. gloeosporioides	38.50±0.19b	54.46±0.18a	29.11±0.08c	30.05±0.18c
F. oxysporum	30.13±0.09ba	47.27±0.26	11.17±0.12d	20.26±0.21c
B. dothidea	100±0.00a	94.87±0.00a	49.51±0.14c	81.22±0.77b
C. cassiicola	100±0.00a	75.12±0.11a	68.10±0.73a	86.81±1.01a

Note: Different lowercase letters indicate statistically significant differences.

3.6 Inhibition effect of cell-free filtrate in pot cultivation

Pot experiment was performed to evaluate the control efficacy of the strains' filtrates.

The control peanut exhibited severe disease incidence with the highest index at 54.32% (Table 4). In cell-free filtrate treatments, the disease indexes were lower with 16.38%-22.84%. For wheat, the cell-free filtrate has no significant positive effect on *F. pseudograminearum* (Table 4).

Table 4. Disease index of each treatment.

	Peanut	Wheat
CK	54.32	54.32
Soil 1-2	32.65	52.47
Soil 1-3	33.27	54.22
Soil 1-59	31.48	54.58
Soil 2-93	37.94	55.38

3.7 Study on peanut growth promotion and defense enzymes

Pot experiment indicated that it is valuable to analysis the changes of peanut defense enzyme activity. Soil 1-2, Soil 1-3, and Soil 2-93 significantly increased fresh weight of above-ground parts with 26%-39% (Figure 5a). Soil 1-2 and Soil 2-93 increased fresh weight of underground with about 59% (Figure 5b). For the dry weight of shoot and root, plant height, and root length, all the strains have no significantly impacts (Figure 5c-f).

The effects of cell-free filtrates on the enzyme activity were shown in Figure 5g-i. The activity of CAT, PAL, and PPO significantly increased by the filtrates of Soil 1-2, Soil 1-3, and Soil 1-59. For Soil 2-93, the cell-free filtrates also increased the activity of CAT and PPO (Figure 5g and 5h), but increased effect for PAL was disappeared after day 1 (Figure 5i).

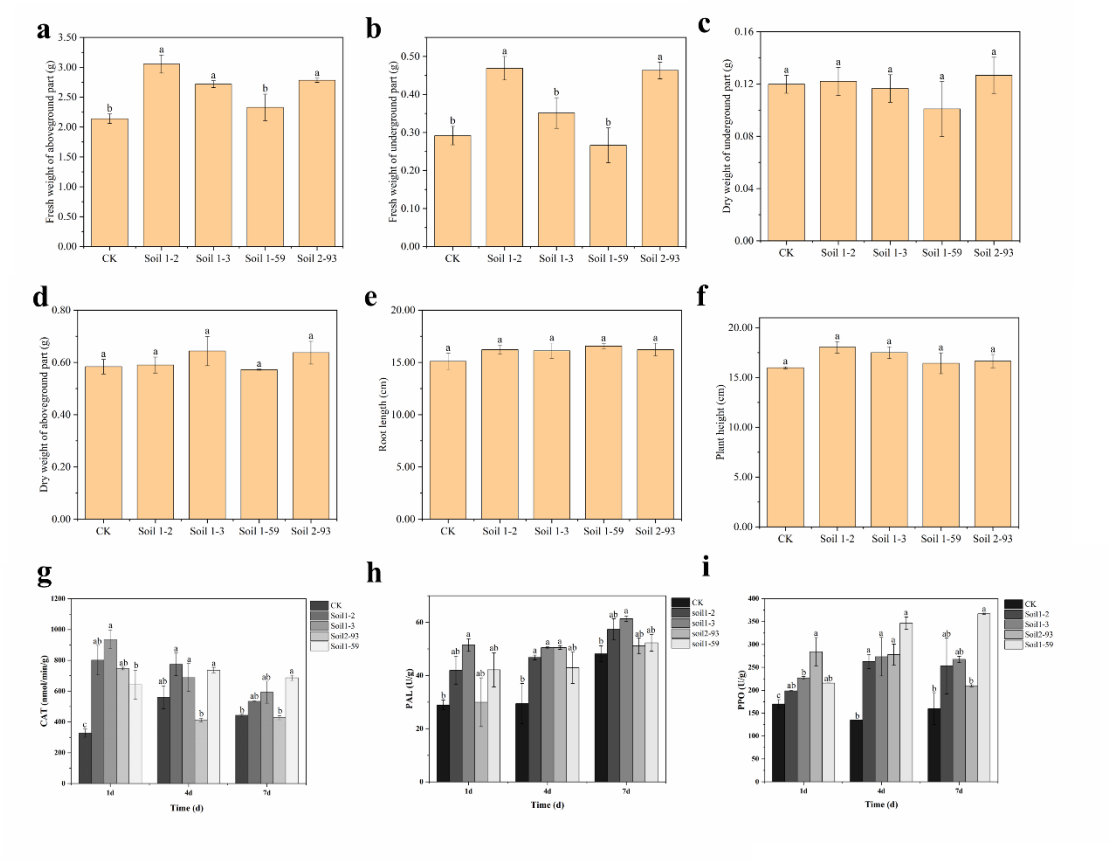

Figure 5. Effect of four bacteria cell-free filtrates on peanut growth and defense enzymes. **Different lowercase letters indicate statistically significant differences.**

3.8 Mechanism of antifungal effect

Considering the potential of the application, two soil-borne diseases pathogens *S. rolfsii*. and *F. pseudograminearum* have been used to carry on the following experiments.

3.8.1 Extracellular enzyme activity

In proteinase medium plate, these four strains all have transparent circles around bacterial colony which indicated that they all have proteinase activities (Figure 6). Iodized solution was added to the starchy plates which has been inoculated and cultivated for 3 days, and transparent circles were formed around the colonies of Soil 1-2, Soil 1-3 and Soil 1-59 (Figure 6). These indicated that the three strains could produce amylase. On cellulose plate medium, the transparent circles showed in the treatments of Soil 1-2, Soil 1-3 and Soil 2-93 after Congo red stain (Figure 6).

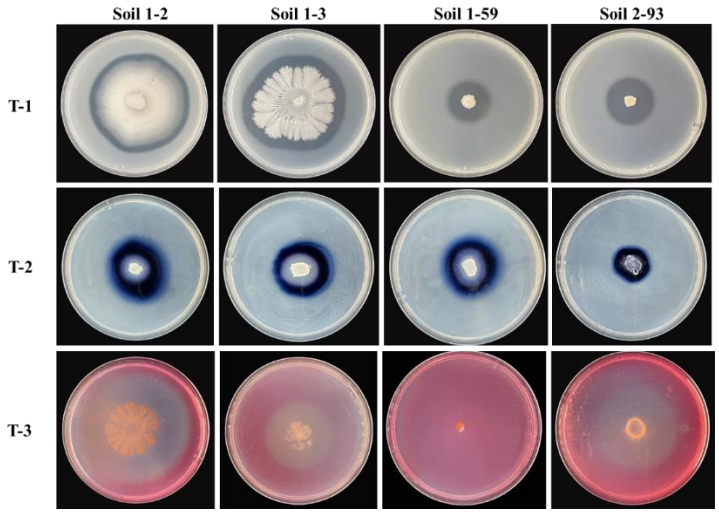

Figure 6. Extracellular enzyme activities of four bacteria. T1 is for protease, T2 is for amylase, and T3 is for cellulase.

3.8.2 Antifungal effect of volatile gases

In plates mouth-to-mouth experiments, the results indicated that volatile gases of these four strains could significantly inhibit *S. rolfsii* growth. Soil 1-3 has the highest inhibition rate with 82.46% (Table 5). And the inhibition rate of Soil 1-2, Soil 1-59, and Soil 2-93 were 74.43%, 68.72%, and 61.64%, respectively (Table 5). But the volatile gases of these strains have limited effect on *F. pseudograminearum*, and only Soil 1-3 and Soil 2-93 showed low effect (less than 9% inhibition).

Table 5. Inhibition rates (%) of antagonistic bacteria volatile gases on *S. rolfsii* and *F. pseudograminearum*

	S. rolfsii	*F. pseudograminearum*
Soil 1-2	74.43±2.81b	-
Soil 1-3	82.64±3.60a	5.86±4.09ab
Soil 1-59	68.72±2.26b	-
Soil 2-93	61.64±1.94c	8.79±3.32a

Note: Different lowercase letters indicate statistically significant differences.

3.8.3 Antifungal effect of crude enzymes

The crude protein solutions of Soil 1-2 and Soil 1-3 have strong inhibition effect on *F. pseudograminearum* with the rate higher than 80% (Table 6). For *S. rolfsii*, the inhibiton rates of Soil 1-2 and Soil 1-3 were 34% and 39%, respectively (Table 6). The inhibition rates of other two strains on both two pathogens all less than 30%

(Table 6). These indicated that the protein of Soil 1-2 and Soil 1-3 could inhibition the pathogen growth.

Table 6. Inhibition rate (%) of antagonistic bacteria crude protein solutions on *S. rolfsii* and *F. pseudograminearum*

	S. rolfsii	*F. pseudograminearum*
Soil 1-2	34.23±2.20ab	79.59±0.00a
Soil 1-3	39.23±3.89a	83.52±0.78a
Soil 1-59	19.51±2.53c	17.24±0.35c
Soil 2-93	29.11±0.62b	27.79±3.13b

Note: Different lowercase letters indicate statistically significant differences.

4. Discussion

In recent years, antagonistic bacteria **attracted** considerable interest in plant protection against various plant diseases. Biological control is a promising method for controlling plant fungal diseases in organic and sustainable agricultural production. Previous reports indicated that a wide range bacterial genera have been documented for plant pathogens control, such as *Bacillus*, *Agrobacterium*, *Arthrobacter*, *Alcaligenes*, *Erwinia*, *Enterobacter*, *Pseudomonas*, *Rhizobium*, *Stenotrophomonas*, *Streptomyces*, *Serratia*, and *Xanthomonas* (Berendsen et al., 2012; Bonaterra et al., 2022). In this work, four *Bacillus* species strains with the ability on controlling plant pathogenic fungi were isolated and identified from the rhizosphere soil of wheat. Confrontation assay on plates indicated that these strains showed broad-spectrum antifungal activity against 9 fungal pathogens. Due to the importance of wheat and peanut, we further conducted research on *S. rolfsii* and *F. pseudograminearum* in terms of disease suppression mechanisms and pot experiment. We found that all the four strains exhibit multiple inhibition mechanisms against the two pathogens.

The pursuit of safe and sustainable strategies for controlling peanut southern blight and wheat crown rot has long been a focus in plant pathology. With advances in biotechnology and increasing environmental concerns, chemical control has gradually lost favor. In its place, the development of resistant cultivars and biological control measures has gained traction. However, resistance breeding is both time-consuming and expensive, as it must be

performed separately for each crop species. By contrast, biocontrol strains offer a rapid and broadly effective alternative, with applicability across different hosts, rendering them a valuable tool for disease management. It is well known that *Bacillus* spp. plays an important role in controlling pathogenic fungi (Bonaterra et al., 2022; Shafi et al., 2017). *Bacillus* spp. is capable of producing a wide variety of antifungal metabolites, which are generally categorized into lipopeptides, proteins, polyketides, and volatile organic compounds (Salazar et al., 2023). **Differences in the types and amounts of antimicrobial substances among these strains are the primary reason for different inhibitory effects against pathogens. In this work, different** inhibition spectrums of these strains also due to this reason. Future studies will focus on elucidating the differences in antimicrobial substance profiles among these four strains.

The results of the extracellular enzyme activity, volatile gases, and crude protein suggested that the antagonistic mechanism of these strains is various. The extracellular enzyme activity of these strains indicated that they could inhibit *F. graminearum* and *P. capsica* through destroying their cell wall (Li et al., 2024). **During growth, microorganisms produce and release various volatile organic compounds into the air, which have inhibitory effects on plant pathogenic fungi. The differences observed between these two pathogens indicate that these volatile compounds were not sensitive to *F. pseudograminearum*. In study of Chaves-López et al.(Chaves-López et al., 2015), they assessed the inhibitory activity of volatiles from 75 Bacillus strains against six fungal pathogens, indicating that *F. oxysporum* and *Moniliophthora perniciosa* were highly susceptible, whereas *Aspergillus parasiticus* showed marked resistance. However, the inhibited effects of the crude enzymes of Soil 1-2 and Soil 1-3 on *F. pseudograminearum* were more sensitive, compared with the effects on *S. rolfsii*. These results indicated that the inhibition mechanism of Soil 1-2 and Soil 1-3 on these two pathogens were different. The inhibited effect of the crude enzymes on fungi indicated that the intracellular enzymes are sensitive to pathogen(Erjaee et al., 2020). That's**

may also the reason why, although the intracellular enzymes of these two strains exhibited strong activity against *F. pseudograminearum*, the cell-free filtrates showed considerable differences in their inhibitory efficacy in the pot experiments against *F. pseudograminearum*.

The increased fresh weight of peanut in treatments indicated that Soil 1-2, Soil 1-3, and Soil 2-93 could promote plant growth (Zhang et al., 2023). In addition, the increased defense enzymes of peanut by cell-free filtrates suggested that these three strains could activate the plants defense capability in adversity. PPO can control diseases by directly generating antibacterial compounds and forming a physical barrier, among other mechanisms (Masood et al., 2008). The primarily function of CAL is to scavenge excessive reactive oxygen species in plants. PAL participates both directly and indirectly in the synthesis of critical plant defense compounds (Zhang et al., 2010). These results illustrated that these three strains have potential to increase peanut growth.

5. Conclusions

This study demonstrated the potential of four *Bacillus* spp. as effective biological control agents against two soil-borne disease peanut southern blight and wheat crown rot. After the initial screening, Soil 1-2, Soil 1-3, Soil 1-59, and Soil 2-93 showed effective inhibition on *S. rolfsii* and *F. pseudograminearum*, and Soil 1-2 and Soil 1-3 has wider inhibition spectrum. Based on the experiments of morphological, biochemical, and molecular analyses, the Soil 1-2, Soil 1-3, Soil 1-59, and Soil 2-93 were identified as *B. velezensis*, *B. subtilis*, *B. atrophaeus*, and *B. pumilus*, respectively. Pot experiments suggested that strain Soil 1-2 and Soil 1-59 exhibited high antifungal activity against *S. rolfsii.*, significantly reducing the degree of disease, enhancing plant health with increased fresh weight and defense enzymes activities. The experiments of volatile gases and crude enzymes indicated that antifungal mechanisms were diverse. Future research should focus on characterizing the secondary metabolites of these strains, identifying the most effective factors on

disease control, and discovering novel antimicrobial compounds.

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