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Streptomyces corchorusii L72 as a potential biocontrol agent against soil born fungi *Sclerotium rolfsii* causing stem rot on peanut

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Abstract *Streptomyces* is a distinctive genus that contains a variety of naturally produced antibiotics and active secondary substances. From 37 actinomyces isolates, isolate L72 was selected because of its highest ability to inhibit *Sclerotium rolfsii* (63.59% inhibition). The antagonistic activity and growth-promoting properties of strain L72 were investigated. This isolate was identified as *Streptomyces corhorsii* L72 based on morphological and physiological properties and analysis of the 16S rRNA gene sequence. Culture filtrate of strain L72 exhibited antagonism activities on mycelial growth and scletorial germination rates of *S. rolfsii* at various diluted concentrations. Interestingly, scletorial germination of *S. rolfsii* was inhibited on the medium with only 2% (50X dilution) of L72's culture filtrate. Biochemical assays revealed that strain L72 produced indole acetic acid, siderophore, and chitinase. The strain also exhibited the ability to solubilize phosphate. Moreover, cell-free culture of this strain promoted peanut fresh weight, root length, and seedling vigor. The data from the pot assay showed that the treatment with *Streptomyces corhorsii* L72 reduced disease incidences when compared with the inoculated control. In conclusion, our results indicated that *Streptomyces corhorsii* L72 was a promising biocontrol agent for controlling stem rot disease.

Keywords: Antifugal activity, Peanut, Sclerotial germination, White stem rot

Introduction

White stem rot disease is the most dangerous disease affecting peanuts globally, caused by the soil-borne fungus *Sclerotium rolfsii* (Mehan *et al.*, 1994). The disease is causing significant economic losses in many different locations and nations (Chen Kun-Rong *et al.*, 2018). *S. rolfsii* has previously posed a significant problem for the production of peanuts by lowering yields and quality (Bot *et al.*, 2011). Wilted leaves and plant damping due to the destruction of

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xylem vessels are the typical symptoms of infected plants (Mullen, 2001). On the decaying plants, the fungus is able to produce a significant amount of sclerotia, which helps it survive in the soil for a long time (Mullen, 2001). Additionally, because of its wide host range, this fungus is difficult to control effectively in the agricultural system. Currently, chemical application is still a successful method for controlling white stem rot (Standish *et al.*, 2019). However, the excessive use of chemicals in agriculture has negative impacts on the environment and human health, as well as encouraging the spread of fungicide-resistant pathogens. Hence, an alternative, environmentally friendly approach such as biological control is needed to control *S. rolfsii* and other fungal plant pathogens.

Among the bacterial community, *Streptomyces* is a unique genus of filamentous bacteria in the Phylum Actinobacteria (Bérdy, 2005). It is well known of their capacity to produce bioactive secondary metabolites, including novel antimicrobial compounds (Sharma and Salwan, 2018). There are many *Streptomyces* species that exhibit antifungal properties against several popular plant pathogenic fungi such as *Fusarium oxysporum* (Li *et al.*, 2021), *Botrytis cinerea* (Ayed *et al.*, 2021), *Rhizoctonia solani* (Wu *et al.*, 2019), and other *C. acutatum C. coccodes, C. gloeosporioides, T. roseum* (Kim *et al.*, 2019). In addition, *Streptomyces* species used as biocontrol agents for controlling *S. rolfsii* have been reported on several host plants. The mechanism of antagonistic *Streptomyces* sp. strains against *S. rolfsii* could be producing antifungal substances (Gebily *et al.*, 2021), cell wall hydrolase enzymes such as chitinase enzyme (Abo-Zaid *et al.*, 2021) or β-1,3-glucanase (Ruangwong *et al.*, 2022), inducing host defense systems (Singh and Gaur, 2017), or plant growth-promoting substances (Jacob *et al.*, 2018). As a result, these *Streptomyces* sp. strains could suppress mycelial growth, sclerotial formation, and germination of *S. rolfsii,* as well as increase plant resistance. According to Jacob *et al.* (2018), using *Streptomyces* sp. RP1A-12 as a biocontrol agent in field trials could decrease the incidence of stem rot disease by 67%. The *Streptomyces* genus is currently a desirable source for discovering novel species with high antagonistic activities and having plant growth stimulants against plant pathogenic fungi. However, there are a limited number of new *Streptomyces* species as biocontrol agents for *S. rolfsii* on peanuts. Therefore, the research focus on *Streptomyces* with strong antifungal activities suppressing peanut stem rot by *S. rolfsii* remains important.

In the present study, strain L72 was isolated from a rhizophere soil sample and exhibited strong antifungal activity against *S. rolfsii* in peanut crops. The objectives were to identify and characterize strain L72, to evaluate the antagonistic effect of this strain on *S. rolfsii in vitro*, and to determine its ability to promote plant growth and suppress *S. rolfsii* in a pot assay.

Materials and methods

Study area

This study was conducted in the laboratory of the Faculty of Biotechnology, Vietnam National University of Agriculture, Vietnam, from September 2021-May 2023.

Isolation and screening of actinomycete strains against S. rolfsii

Actinomycete strains were isolated from rhizosphere soil samples of vegetable crops in Gia Lam, Hanoi, Vietnam, using the serial dilution method on Gause's No. 1 medium (30^{o}C, 7 days). Isolates were screened for antifungal activity against *S. rolfsii* by the co-culture method at 30^{o}C. Plates without the actinomycete were used as control plates. Fungal growth inhibition (%) of the tested strains was calculated according to the following formula (i):

$$I\% = \frac{C-T}{C} \text{ x } 100 \text{ (i)}$$

C and T are fungal colony radius in control plates and in co-culture plates on the opposite side of actinomycete, respectively.

Identification of actinomycete strain L72

The genomic DNA of the actinomycete was extracted according to the modified CTAB method. The 16S rRNA gene of the isolate was amplified from purified DNA using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACC TTGTTACGACTT-3') primers. The amplified DNA samples were sequenced and compared with other related sequences using the BLAST tool (http:/www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic relationships were determined using MEGA 6.0 for the strain-related species retrieved from GenBank.

Physiological and biochemical studies

Cultural characteristics of strain L72 were evaluated on ISPs and Gause media (Shirling and Gottlieb, 1966). The growth of strain L72 was also studied at different temperatures (25-50^{o}C), pH ranges (4-12), and NaCl concentrations (0-5%) on Gause's No. 1 medium for 7-14 days. Other biochemical properties of the strain L72, including indole acetic acid, siderophore, chitinase production, and phosphate solubilization were investigated as described by Abbasi *et al*. (2019).

Evaluating the effect of strain L72 on mycelial morphology, growth, and sclerotial germination rate of S. rolfsii

The effect of strain L72 on mycelial morphology was evaluated under microscopy by co-culturing its culture filtrate with *S. rolfsii* on PDA for 72 hours (Taechowisan *et al.*, 2009).

In the growth inhibition assay, the strain L72 was inoculated in Gause's No. 1 broth at 30°C for 7 days. Next, the culture was filtered through a 0.2 µm membrane. Then the filtrate was diluted with sterilized water in proportions of 1:0, 1:4, and 1:9 (*v*/v) before being mixed with PDA medium in a ratio of 1:4 (*v*/v). Fungal discs (5 mm) were placed at the center. The diameter of the fungus was measured after 24, 48, and 72 hours of incubation. The hyphal growth inhibition was calculated using formula (ii):

$$\text{IRMG\%} = 1 - \frac{\text{Dt}-5}{\text{DCK}-5} \text{ x } 100 \text{ (ii)}$$

IRMG is the rate of hyphal growth inhibition. DCK and Dt are the diameters of fungal colonies on PDA without and with the filtrate, respectively (Zhao *et al.*, 2012).

To determine the inhibition of *Streptomyces* sp. L72 on sclerotial germination, *S. rolfsii* sclerotia were transferred to PDB medium containing the culture filtrate. The culture filtrate of *Streptomyces* sp. L72 was diluted and added to PDB (ratio 1:4 v/v) to archive the final concentrations as 5X, 25X, and 25X. After 3 days of culturing, the mycelial biomass was collected. The sclerotial germination inhibition rate was calculated and compared (Li *et al.*, 2017).

Testing the effect of S. corchorusii L72 culture filtrate on peanut seedlings

To determine the effect of *Streptomyces* sp. L72 on peanut seedlings, the peanut seeds were surface-sterilized and soaked in the culture filtrate for 2 hours, while water treated seeds were used as the control. The seeds were placed in a sterilized plate containing wet filter papers. After 2 weeks of incubation at 30°C, the lengths, fresh weight, dry weight of shoot/root, and vigor index were collected (Jacob *et al.*, 2018).

Pot assay for testing the biocontrol ability of S. corchorusii L72

To test the biocontrol ability of *S. corchorusii* L72 against *S. rolfsii*, a pot assay with the peanut variety ICGV98369 was conducted. *S. rolfsii* innoculum was prepared with a 5x5 mm fungi block cultured in 60 ml PBD medium for 5 days, then diluted with 120 ml of sterile water. 15-day-old peatnut seedlings were grown in pots with sterilized soil for this study. Two treatments were set up as

follows: inoculating pots with 10 ml of *S. corchorusii* L72 cultured 7 days before infection with 5 ml of *S. rolfsii,* and co-inoculating *Streptomyces* sp. L72 culture (10 ml) and *S. rolfsii* (5 ml). The control was inoculated with *S. rolfsii* (5 ml) and distilled water (10 ml). After 20 days of infection, the plants were uprooted, and the fresh and dry weights of the plants were collected. Percent disease incidence (PDI) and percent disease control (PDC) were collected according to Rakh *et al* (2011).

Data analysis

All experiments were repeated three times, and the data were expressed as the mean ± standard deviation (SD). Statistical analysis was subjected to one-way ANOVA using GraphPad Prism 8.0.

Results

Isolation of actinomycete strains against S. rolfsii

From collected soil samples, six out of thirty-seven actimomycete isolates with antagonistic activities against *S. rolfsii* were obtained. Based on their characteristics, an isolate (strain L72) exhibited the highest antagonistic activity with mycelial inhibition of 63.59 ± 1.78 % (Figure 1A-B). Beside that, the culture filtrate of strain L72 showed an effect on the morphology of mycelium *S. rolfsii* (Figure 1C–D). The mycelia of *S. rolfsii* were distorted and coiled when cultured on medium containing culture filtrate of strain L72.

Identification of actinomycete strain L72

The strain L72 grew well on all tested Gauses and ISP media. The color of substrate mycelium and aerial mycelium is different on most media (Table 1). This strain has no dark-soluble pigment (melanoid) on ISP6 medium. SEM analysis revealed that the strain L72 produced a long chain of spore production as a spiral type (Figure 2B). In addition, the sequencing of the 16S rRNA gene of strain L72 showed the highest similarity level of 98.99% for *Streptomyces corchorusii* AUH-1. The neighbor-joining phylogenic tree was constructed, indicating strain L71 was closely related to *Streptomyces corchorusii* (Figure 3). Based on its morphological, physiological, and molecular characteristics, strain L71 was identified and named *Streptomyces corchorusii* L72.

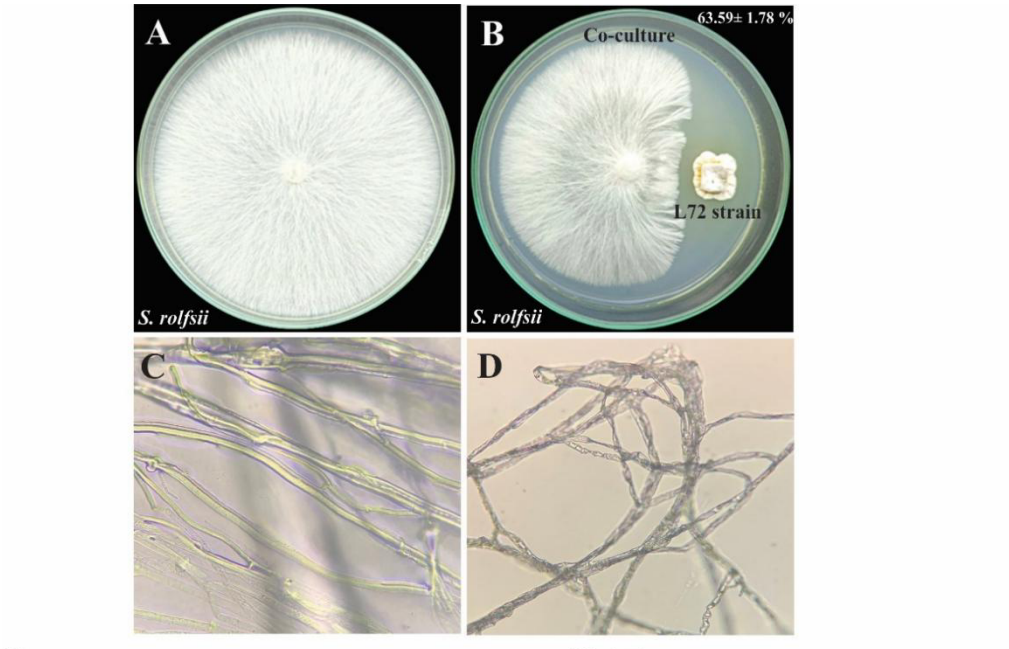

Figure 1. Antagonistic activity of strain L72 against *S. rolfsii*: (A-B) The growth of *S. rolfsii* on the control plate (only *S. rolfsii*) and on the treatment plate (inoculated with *S. rolfsii* and strain L72) for 5 days; (C-D) Mycelial morphology of *S. rolfsii* on the control (C) and on the treatment with culture filtrate of *actinomycete strain* L72 (D).

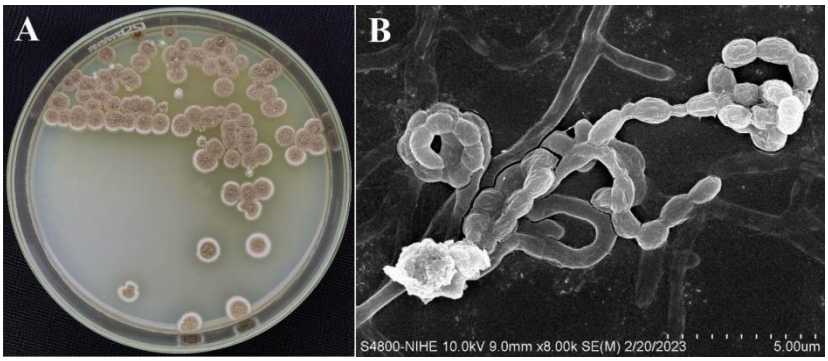

Figure 2. Colony morphology of strain L72 on ISP3 (A) and strain L72's spore chain under scanning electron microscopy (B)

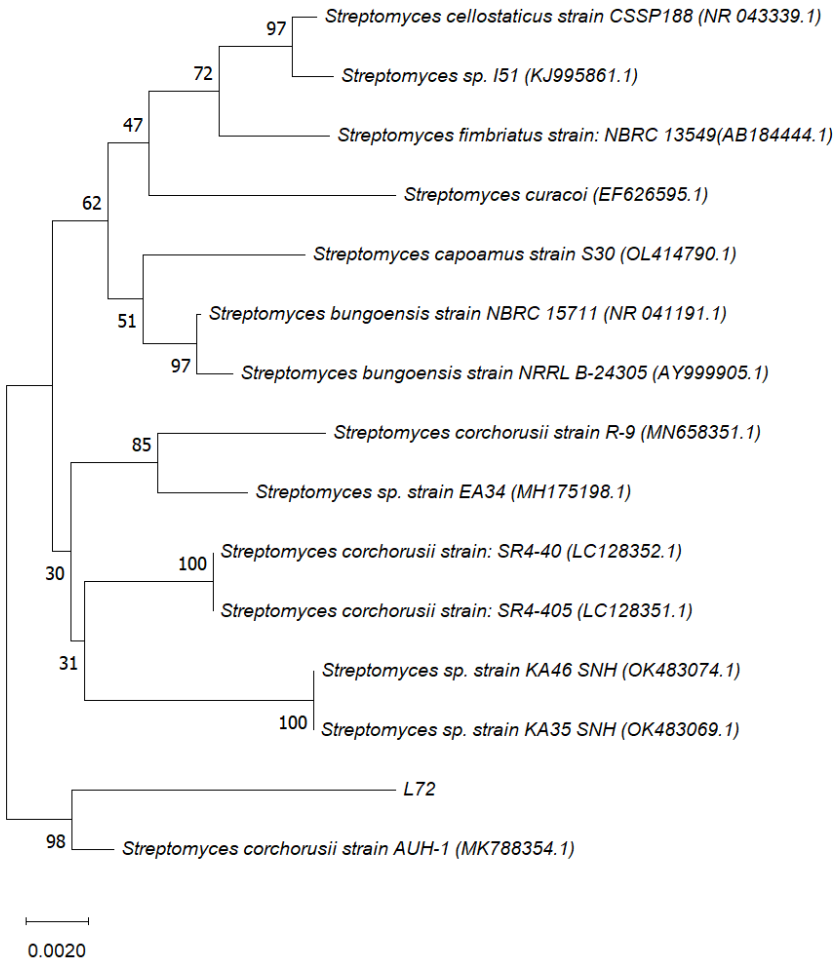

Figure 3. Neighbour-joining tree based on 16S rRNA gene sequence, showing the relationships between L72 and other related strains of *Streptomyces* species

Characteristics of Streptomyces corchorusii L72

Testing the physiological and biochemical characteristics revealed that *S. corchorusii* L72 was able to grow in a wide range of temperatures (25-50^{o}C) and pH of media (4-12). The strain can utilize fructose, sucrose, or D-glucose as carbon sources and meat extract or pepton as nitrogen sources. Interestingly, the strain *S. corchorusii* L72 exhibited plant-growth-promoting properties, including utilizing insoluble phosphate, producing IAA and sidorephore production. In addition, this strain displayed strong chitinase activity (Table 1).

Table 1. Physiological, biochemical and nutrition utilization characteristics of *S. corchorusii* L72

Characteristics	Results (optimal conditions)
Temperature range for growth (°C)	25-50 °C (optimal growth at 30-40°C)
pH range for growth	4-12 (optimal growth at pH 6-9)
NaCl tolerance for growth (%)	0-5%
IAA production	4.94 µg/ml (5 days of culture)
Siderophore production	11 mm (after 15 days of incubation)
Phosphate solubilization	17 mg/mL (after 7 days of incubation)
Carbon source utilization	Fructose/Sucrose/D-glucose
Nitrogen source utilization	Meat extract/Peptone
Chitinase (halo zone)	19 mm

Effect of S. corchorusii L72 *culture filtrate on S. rolfsii*

S. corchorusii L72 culture filtrate inhibited the mycelial growth of *S. rolfsii* in all three selected diluted concentrations (50X, 25X, and 5X). Compared to the growth diameter of *S. rolfsii* in the control plate at different time points, growth inhibition was clearly observed in the treatments with culture filtrate of *S. corchorusii* L72. At 72 hours of treatment, the inhibitory efficiency was 4.70%, 8,23%, and 39,60% in the three tested concentrations (Figure 4A). Similarly, the culture filtrates of *S. corchorusii* L72 also inhibited the hyphal growth from sclerotia with inhibition rates of 7.78% (50X), 12.86% (25X), and 16% (5X).

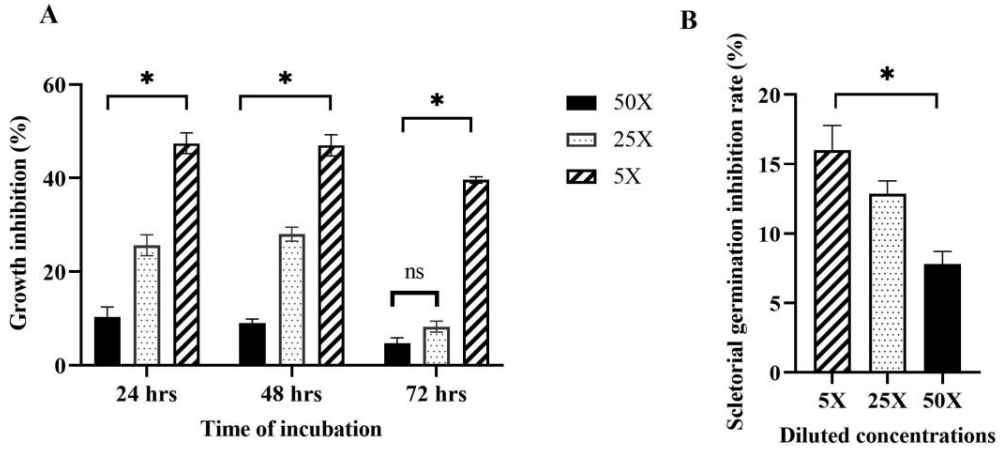

Figure 4. Effect of the strain L72's culture filtrate on the growth (A) and sclerotial germination rate of *S. rolfsii* (B) at different concentrations. * indicates a significant difference ($p< 0.05$) between different treatments at the same time; ns means no significant.

Effect of S. corchorusii L72 culture filtrate on peanut seedlings

The treatment with culture filtrate of *S. corchorusii* L72 increased the fresh weight and root length of peanut seedlings compared with the controls ($p < 0.05$) as shown in Table 3. However, no significant difference was observed in the dry weight and shoot length of seedlings between control and treatment. Notably, the vigor index in the treatment was significantly higher than the control. Thus, the culture filtrate of *S. corchorusii* L72 had a growth-promoting effect on peanut seedlings.

Table 3. Effect of *S. corchorusii* L72 on peanut seedling growth (after 2 weeks)

Treatment	Fresh weight ± SD (mg)	Dry weight ± SD (mg)	Root length ± SD (mm)	Shoot length ± SD (mm)	Vigor index
Control (water)	974 ± 66.4[a]	154.1 ± 21.8[a]	79.1 ± 19.8[a]	32.9 ± 5.4[a]	996.9 ± 199.7[a]
Inoculated with *S. corchorusii* L72	1211 ±147[b]	231.6 ± 24.4[a]	93.4 ± 12.5[b]	40.7 ± 3.9[a]	1231 ± 128.7[b]

Means followed by a different letter in the same columns are significantly different at $p<0.05$.

Effect of S. corchorusii L72 against S. rolfsii in vivo

The results of testing the ability of strain L72 against *S. rolfsii* are summarized in Figure 5. The disease incidence of two treatments with application of *S. corchorusii* L72 was significantly decreased when compared with the control treatment (Figure 5A). There was no significant difference in disease incidence between the ways of treating soil with *S. corchorusii* L72 before or at the same time of *S. rolfsii* infection. Therefore, the percent disease control of these two treatments was also reduced to levels of 50-63.63% and 40.90-52.17%, respectively.

Discussion

Plant diseases caused by pathogenic fungi are one of the main factors that reduce agricultural production. The biocontrol strategy is an alternate method for reducing the usage of fungicides, fertilizers, and herbicides (Jaiswal *et al.*, 2022). This strategy is considered the most promising solution for sustainable agriculture (Jaiswal *et al.*, 2022). Interestingly, actinomycetes are important biological resources due to their capacity to produce novel secondary metabolites (Bérdy, 2005). As a result, recent research has focused on *Streptomyces* species

with antagonistic abilities to protect plants from diseases. In the current study, the antagonistic strain of *Streptomyces* against *S. rolfsii*, *S. corchorusii* L72, was isolated and identified based on morphology and the nucleotide sequences of 16S rDNA. The strain L72 exhibited strong antifungal activities in both *in vitro* and *in vivo* conditions.

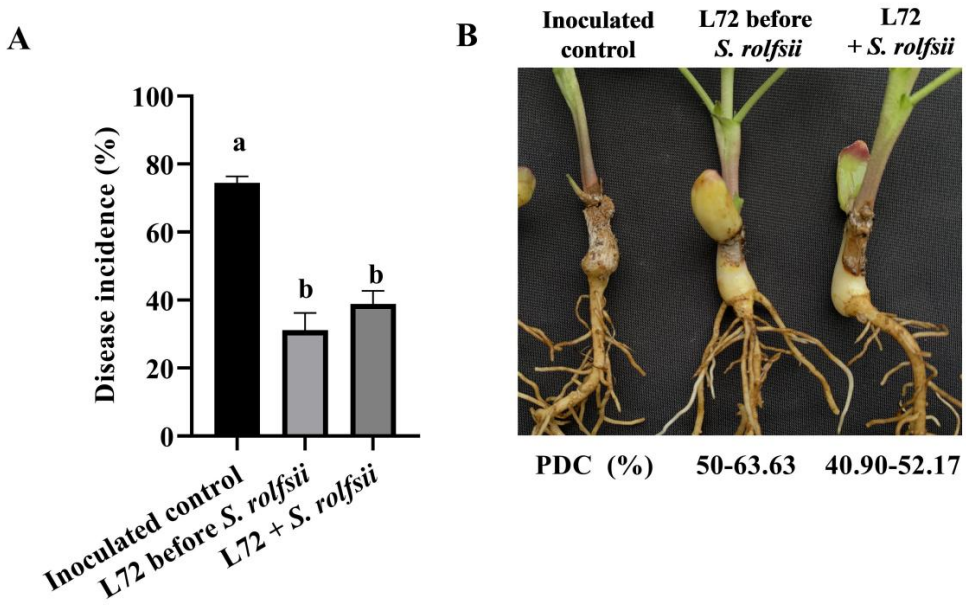

Figure 5. Antifungal activity of *S. corchorusii* L72 against *S. rolfsii in vivo* condition: (A) Disease incidence; (B) Disease symptoms of peanut stem; Inoculated Control (only *S. rolfsii*); L72 before *S. rolfsii* (Innoculated with strain L72 for 7 days followed by *S. rolfsii)*; L72 + *S. rolfsii* (Innoculated with L72 and *S. rolfsii* at the same time).

The antifungal effect of strain L72 on *S. rolfsii* was demonstrated by the effects of culture filtrate on mycelial growth, hyphal morphology, and spore germination rate. Culture filtrate of *S. corchorusii* L72 strongly suppressed mycelial growth of *S. rolfsii* in different diluted concentrations. Additionally, treating fungal mycelium with the culture filtrate of *S. corchorusii* L72 resulted in mycelial deformation, coiling, and distortion. The culture filtrate also inhibited *S. rolfsii* spore germination rate. Interestingly, *S. corchorusii* L72 could produce the chitinase enzyme, indicating the ability to decompose the fungi's cell wall. These results suggested that the antagonistic activities of this strain against *S. rolfsii* may be due to its antifungal metabolites and cell wall degrading enzymes.

Our findings are consistent with other studies. Recently, several *Streptomyces* species have revealed the potential of inhibiting *S. rolfsii* as biocontrol agents. The strain *Streptomyces albulus* Z1-04-02 could suppress *S. rolfsii* with the production of cell wall degrading enzymes (CWDEs), antifungal metabolites, and volatile compounds (Ruangwong*et al.*, 2022). Other species, such as *S. griseus*, *S. rochei*, and *S. sampsonii,* inhibited mycelial growth, sclerotia germination by antifungal substances (Gebily *et al*., 2021). *Streptomyces cellulosae* Actino 48 exhibited strong inhibition against *S. rolfsii* and had high chitinase enzyme production. (Abo-Zaid *et al.*, 2021). In addition, research on *S. corchorusii* also demonstrated the antifungal activities of this *Streptomyces* species on other pathogenic fungi. The *S. corchorusii* strain UCR3-16 is reported to have strong antifungal activities against six fungal pathogens by producing several fungal CWDEs such as chitinase, β-1,3-glucanase, β-1,4-glucanase, lipase, and protease (Tamreihao *et al.*, 2016). In another report, *S. corchorusii* strain AUH-1 exhibited broad-spectrum antifungal activity on several fungi, and its antifungal metabolite could damage fungal cell membranes (Yang *et al.*, 2019).

Interestingly, *S. corchorusii* L72 exhibited important plant growth-promoting traits, including IAA, siderophore production, and P solubilization. In addition, culture filtrate of *S. corchorusii* L72 promoted seedling growth and showed a significantly higher vigor index compared to control seedlings. The creation of IAA and siderophore may account for this strain's capacity to encourage seedling growth. Similarly, multiple *Streptomyces* species have been reported to have both antagonistic and plant growth promoting traits (Kaur and Manhas, 2014; Li *et al*., 2021). In addition, the present study showed that strain L72 had significant antagonism activity against *S. rolfsii* in a pot assay. The disease incidences in treatments with *S. corchorusii* L72 were significantly decreased indicating this strain could suppress *S. rolfsii in vivo*. Overall, our data indicated that *S. corchorusii* strain L72 is a potential and promising candidate as a biocontrol agent against *S. rolfsii.* However, more study on this strain, including as field experiments, is required before it is recommended to be used as a biocontrol agent.

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Supplemental Table 1. Cultural characteristics of strain L72 on different media

Medium	Growth[/1]	Substrate mycelium	Aerial mycelium
Gause's No. 1	+++	White	Yellowish
Gause's No. 2	++	White	Yellow
ISP1	++	White	White
ISP2	+++	White	Brown
ISP3	+++	Gray	Brown
ISP4	+++	Brown	Brown
ISP5	++	White	Yellowish
ISP6	++	White	White
ISP7	++	Gray	Gray

[/1] *+++: strong growth, ++: moderate growth*

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