

# Antifungal Activity of *Streptomyces flaveus* against *Fusarium solani* Isolated from the Dry Rot Potato

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Dry rot of potatoes is caused by several fungal species in the genus *Fusarium*, of which *Fusarium solani* is an outstanding cause of postharvest potato losses globally. The fungus can also cause a broad spectrum of infections in humans and animals. The dry rot disease spread in the potato field has been treated mainly using fungicides that are very harmful to human health. The use of fungicides also pollutes the living environment and unbalances the microbial ecosystems in nature. *Streptomyces flaveus* has been known to produce antibiotic SW-B capable of inhibiting the growth of several plant pathogenic fungi. However, it has not been clear whether *S. flaveus* is able to inhibit the growth of *F. solani*. This study isolated the fungus that caused the potato dry rot from the diseased potato tubers and evaluated the antifungal activity of *S. flaveus* against the isolated fungus. Cell morphology and phylogenetic analyses of the fungus showed that *F. solani* was successfully isolated. It was found that this fungal isolate was sensitive to *S. flaveus*. This actinomycete produced extracellular chitinase whose concentration in the liquid culture was affected by the supplementation of colloidal chitin and fungal cell powder into the culture medium, making the *F. solani* mycelial cell an important factor for optimizing chitinase concentration in biocontrol preparation for prevention and treatment of the dry rot disease in potato tubers.

## 1. Introduction

Potatoes are of very high nutritional values and are safely consumed all year round not only in European countries but also in many other countries around the world. However, the development of economy and global warming has favored the development of the potato dry rot disease. The disease is caused by different species belonging to genus *Fusarium* of which *Fusarium solani* is the main agent. This microorganism not only seriously reduces the quality and yield of potatoes but also causes a broad spectrum of infections in humans (Nucci and Anaissie, 2007) and animals (Evans et al., 2004). The infection is considered very difficult to treat because of the lack of protocols for treatment of multi-drug resistant strains (Batista et al., 2020). *F. solani* has also been of great concern because the prevention and control of the growth and spread of this pathogen have not been well established. The prevention of the disease has so far been relied mainly on good care and proper storage of tubers by good farmers such as not harvesting potatoes for seeding on wet days, avoiding scratching during transportation of potatoes, stacking the tubers after harvest into separate thin layers for drying before being put in storage, keeping the storage conditions dry and cool, monitoring and checking the stored tubers regularly to eliminate the diseased tubers; other good management practices such as crop rotation, timing of planting or using disease-free bulbs for cultivation have also been implemented. Alternatively, the disease has also been treated mainly using fungicides. <sup>BM</sup>Bordeaux M 25WP is an antifungal preparation that is commercially available for making bordeaux solutions at different concentrations ready for spraying. It is essentially a mixture of calcium hydroxide and copper sulfate. Copper ion can damage the fungal cell membranes and react with the reactive oxygen species intracellularly (Pariona et al., 2019) due to its oxidation ability. Therefore, copper is also toxic to other useful microorganisms and plants. When consumed in excess, copper can lead to oxidative stress, accelerated cognitive decline and increased risk of alzheimer's disease in humans (Coelho et al., 2020). Other commercial preparations that have been used to chemically treat the potato dry rot disease are Kolfugo Super, Bavistin 50SL, Carbezim 50EC, etc. The active component in these preparations is carbendazim, which can cause embryotoxicity, cell death, teratogenicity, infertility, liver-cell dysfunction, endocrine dysfunction and

disrupt the microbial communities in different ecosystems (Singh et al., 2016). Carbendazim acts by binding and thus preventing the protein tubulin of the *Fusarium* species from polymerization (Tarafder and Datta, 2022), which in turn leads to the lack of microtubules which function in many processes, including structural support, intracellular transport, and DNA segregation. Other chemicals that were found to effectively inhibit the growth of *F. solani* includes thiabendazole, fludioxonil, azoxystrobin, difenoconazole, chlorothalonil (Tiwari et al., 2020), mancozeb (Ghimire et al., 2021), metalaxyl (Satyanarayana et al., 2021), etc. Preparations containing these chemicals such as Tan Qui Talaxyl 25WP, Manozeb 80WP, etc. are commercially available. The excessive use of these chemical fungicides has contaminated drinking water sources (Marucco et al., 2017), reduced the abundance and diversity of soil and aquatic microorganisms and animals, thus beside continuous monitoring of water and soil, the development of eco-friendly bio-fungicides has been proposed (Rad et al., 2022). A number of useful microbes such as *Pseudomonas*, *Enterobacter*, *Pantoea*, *Trichoderma*, *Bacillus*, etc. have been reported to be capable of controlling the dry rot in potatoes (Tiwari et al., 2020). However, microbial preparations that are capable of inhibiting *F. solani* are rare. Bactofit® is one of the rare preparations commercially recommended for preventing the dry rot disease caused by *F. solani* in potatoes. It contains *Bacillus subtilis*, a harmless bacterium that can compete with many other microorganisms for nutrients. It also secretes a lot of extracellular lipopeptides to suppress the growth of *F. solani* and promotes the growth of plants (Qiao et al., 2023). *Streptomyces flaveus* has been found to be able to suppress the growth of *Colletotrichum truncatum* – a chili anthracnose pathogen, and *Neoscytalidium dimidiatum*, a dragon fruit plant pathogen, due to the fact that it possesses the enzyme chitinase (Phan et al., 2023). It has not been clear so far whether *S. flaveus* is able to suppress the growth of *F. solani* and whether its chitinase would interact with this fungus. This research aimed to isolate *F. solani* from the dry rot potatoes and investigate the antifungal activity of *S. flaveus* against the isolated fungus for a safe and sustainable method of controlling the potato dry rot disease.

## 2. Materials and Methods

### 2.1 Fungal isolation

Potato tubers with dry rot symptoms were collected from a vegetable garden in October 2022, in Dalat City, Lam Dong Province, Vietnam. The diseased tuber surface was wiped with a cotton swab soaked in the solution of 70 % ethanol. The tuber was then dipped into the solution of 1.5 % sodium hypochlorite for 3 min, placed on the absorbent paper and allowed to air-dry. Pieces of 5 -10 mm in size containing mycelia were cut from the tuber and placed on the potato-dextrose agar (PDA) containing plates. The plates were incubated in a black, sealed nylon bag at 25 °C. After 3 days of incubation, when more mycelia growing on the agar surface, an agar slice containing aerial mycelia was cut and put into a cup of sterile distilled water. The water was diluted and 10 µL of the diluted water was spread on the PDA plates. The plates were incubated at 25 °C until the mycelia were observed separately on the agar surface. The distinct mycelia were repeatedly streaked onto new PDA plates until pure culture was obtained. The pure culture was regularly examined microscopically. A strain with distinct characteristics for *F. solani* was suspected to be the cause of the dry rot and used to infect the fresh healthy potatoes for observing the dry rot symptoms.

### 2.2 Artificial infection

Healthy potato tubers were washed under the tap water and then subsequently dipped into the solution of 70 % ethanol for 30 s and the solution of 1.5 % sodium hypochlorite for 3 min, then rinsed with distilled water for 10 min. A metal needle was used to puncture a hole in the potato tuber about 2 cm in depth. The hole was then inoculated with pure mycelia of the suspected dry rot causing strain and covered with a small pad of sterilized waterproof cotton. The cotton pad was gently secured with an elastic band. The inoculated tubers were wrapped inside a black nylon bag and incubated at 25 °C for 20 days. The tubers after that were examined for the dry rot symptoms.

### 2.3 Fungal identification

Identification of the isolated fungal species at the molecular level was carried out at the Molecular Diagnostics Department, Nam Khoa-Biotek Laboratory (License number 05505/SYT-GPHD), Ho Chi Minh City, Vietnam. Genomic DNA was extracted and used as template for polymerase chain reaction (PCR) with the primers specific to the ribosomal DNA (rDNA) region. The resulting PCR product was then sequenced and blasted against the National Center for Biotechnology Information (NCBI) database (<https://blast.ncbi.nlm.nih.gov>). The rDNA fragments which were closely related to the PCR sequence were downloaded from the database and used for the phylogenetic analyses, using the MEGA11 software (Tamura et al., 2021). Muscle program in the software was used for multiple sequence alignment. Neighbor - Joining method was used for the construction of the phylogenetic tree with 1,000 times of bootstrapping.

## 2.4 Antifungal activity test

The fungal spores were inoculated onto the center of a PDA plate. The spores of *S. flaveus* (Phan et al., 2015) were also streaked at four surrounding positions within the plate, about 2 cm from the center. The plate was inverted and incubated at 30 °C for 7 days. Transparent areas around the actinomycete hyphal mats on the agar surface, if present, were recorded. A plate inoculated with the fungus only was used as control. For testing the ability of the extracellular metabolites produced by the actinomycete to suppress the fungal growth, *S. flaveus* spores were inoculated into 25 mL of starch casein broth (SCB) medium contained in a 250 mL conical flask. Composition of the SCB included soluble starch (10 g/L), casein (0.3 g/L), KNO<sub>3</sub> (2 g/L), NaCl (2 g/L), K<sub>2</sub>HPO<sub>4</sub> (2 g/L), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.05 g/L), CaCO<sub>3</sub> (0.02 g/L), and FeSO<sub>4</sub>·7H<sub>2</sub>O (0.01 g/L), pH = 6.8 ± 0.2. The flask was shaken at 30 °C, 120 rpm for 30 h on an orbital shaker. Then, 0.75 mL of the culture broth was transferred into another 250 mL conical flask containing 25 mL of SCB. The flask was shaken on the orbital shaker at 120 rpm and 30 °C for 4 days. The culture broth was then centrifuged at 4,000 rpm for 20 min to obtain the supernatant. 100 µL of the supernatant was pipetted into the 8 mm-wells drilled on the PDA plates spread with the fungal spores. Culture broth which contained the actinomycete's cells, sterile SCB and sterile distilled water were used as controls. The plates were incubated for 3 days at 25 °C and were checked for the appearance of clear zones around the wells. *In vitro* chitinase test for the presence of chitinase in the *S. flaveus* supernatant was carried out as described previously (Phan et al., 2023).

## 2.5 Fungal cell powder preparation

*F. solani*'s biomass was prepared as done previously (Phan et al., 2023). However, the liquid culture was shaken at 25 °C and the cell pellet was collected by centrifugation at 4,000 rpm for 20 min. The pellet was washed three times with distilled water before being suspended in distilled water and spreaded evenly on a plate. The plate containing the fungal cell pellet was dried completely at 70 °C. The fungal cell powder was obtained by scraping the remaining dried fungal biomass on the plate.

## 2.6 Chitinase production

The *S. flaveus* spores were inoculated as long streaks of about 2.5 × 0.3 cm in size on the colloidal chitin agar and chitin casein agar plates. The chitin casein agar was the same as the starch casein agar but 10 g/L of soluble starch was replaced by colloidal chitin 1 % (v/v). The plates were incubated at 30 °C for 4 days, then covered with Lugol's solution 1 % to examine for the clear zones around the *S. flaveus* hyphal mats.

The colloidal chitin and fungal cell powder were supplemented into the SCB medium at the ratios of 1:1, 1:2 and 1:3 such that their total concentration in the medium was 10 g/L. The *S. flaveus* spores were inoculated and cultured as described previously (Phan et al., 2023). SCB medium without supplementing the colloidal chitin and fungal cell powder was also run in parallel to the supplemented media. Collection of the culture broth samples was carried out as done previously (Phan et al., 2023).

## 2.7 Comparison of chitinase production

The *S. flaveus* supernatants obtained from the culture broth samples were used for determination of changes in chitinase production during the culture time, using 3,5-dinitrosalicylic acid (DNS) as described as before (Phan et al., 2023). The supernatant of the fresh medium that was used for chitinase production was prepared in parallel as blank. Each sample was carried out in triplicates. Two-way analysis of variance (ANOVA) test was conducted, followed by Tukey's test using the Excel add-in Real Statistics package, at p = 0.05.

## 3. Results and Discussion

### 3.1 Isolation and identification of the potato dry rot causing agent

From the dry rot diseased potato tuber (Figure 1a), three different strains of filamentous fungi were isolated, one of which was suspected to be the dry rot causing agent. It grew rapidly on PDA at 25 °C, formed porous and flat cottony colonies on the agar surface (Figure 1b). Its hyphae were septate, branched and hyaline. Its microconidia were cylindrical and end-rounded while its macroconidia were curved and end-pointed (Figure 1c). It also had rounded and thick-walled chlamydospores (Figure 1d). These morphological characteristics were also observed for many different strains of *Fusarium solani* previously (Chehri et al., 2015). When inoculated back into a fresh healthy potato tuber, after incubation, the inside of the tuber formed a large cave filled with cottony white mycelia (Figure 1e).

After cloning the rDNA sequence from the fungal isolate's genome using PCR, the PCR product was sequenced and a sequence of 843 bp was obtained. A blast search against the NCBI database showed that the sequence was highly similar to the region encoding a part of 18S ribosomal RNA (rRNA), 5.8S rRNA, and a part of 28S rRNA of many *Fusarium* species. The phylogenetic tree constructed from a number of the related rDNA

sequences is shown in Figure 2. The tree shows that the isolate that caused the potato dry rot, called *F. solani* DL, has very close relationships with several other known *F. solani* strains.

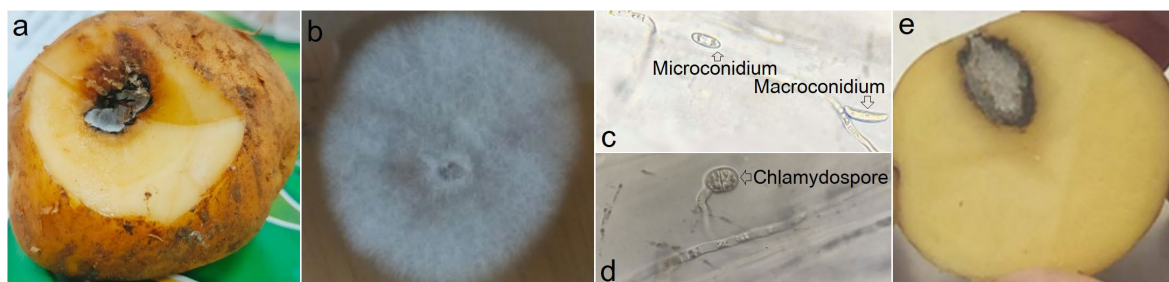


Figure 1: (a) The dry rot symptoms inside of a potato tuber from the vegetable garden; *F. solani* DL mycelia on (b) PDA and (c, d) under the light microscope at 400x resolution; (e) A large cave formed by artificial infection

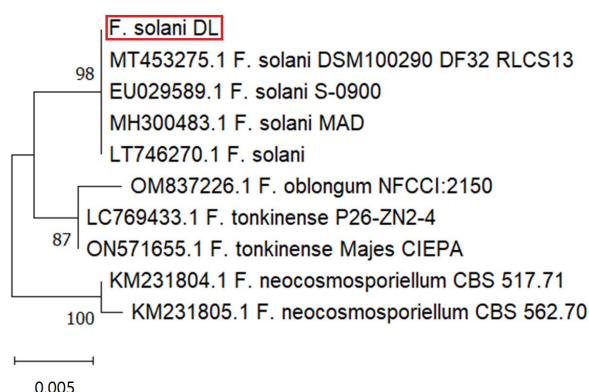


Figure 2: Phylogenetic relationships among the *Fusarium* species. Numbers at the nodes are the bootstrapped values. The NCBI accession numbers are shown before the species names

### 3.2 Antifungal activity of *S. flaveus* against *F. solani* DL

When *F. solani* DL was co-cultured with *S. flaveus*, it was found that this fungal isolate was sensitive to *S. flaveus*. It can be seen from Figure 3a that the fungal growth was inhibited in the zones around *S. flaveus*'s hyphal mats. This means that one or more substances in the supernatant that had diffused into the agar inhibited the growth of *F. solani* DL, while on the agar without the presence of *S. flaveus*, *F. solani* DL grew freely (Figure 3b).

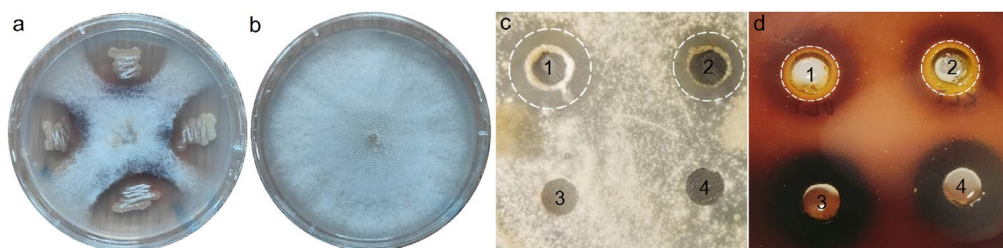


Figure 3: *F. solani* DL's growth inhibited by (a) *S. flaveus* on PDA and (b) *F. solani* DL control. (c) Growth of *F. solani* DL around the wells with (1) *S. flaveus* culture broth, (2) *S. flaveus* supernatant, (3) SCB and (4) distilled water. (d) Degradation of colloidal chitin around the wells with (1) *S. flaveus* supernatant, (2) *S. flaveus* culture broth, (3) SCB and (4) distilled water

Figure 3c shows that these fungal inhibiting substances were extracellular because the transparent areas appeared around the wells added with the *S. flaveus* culture broth and supernatant. The transparent area around the well added with the *S. flaveus* culture broth was larger than that added with the supernatant. This was perhaps due to more fungal inhibiting metabolites produced by the actinomycete, which continued to grow on the PDA during the incubation time, diffused into the agar medium.

To check if chitinase was the factor that had diffused into the agar medium, the colloidal chitin agar was used to show the presence of this enzyme in the agar medium. The experiment verified that chitinase from the *S. flaveus* supernatant and culture broth indeed diffused into the agar medium and degraded the colloidal chitin present in the agar medium (Figure 3d).

### 3.3 Effects of the colloidal chitin and *F. solani* cell powder on chitinase production by *S. flaveus*

It was found that the chitinase production by *S. flaveus* was affected by the medium composition. On the chitin casein agar, *S. flaveus* secreted more chitinase than on the colloidal chitin agar (Figure 4a and b). The clear zones around the hyphal mats on the former medium (Figure 4b) look larger and brighter than those on the later medium (Figure 4a), as the more chitinase was present in an area of the agar medium, the more colloidal chitin present in that area was degraded so no chelation between  $I_2/KI$  in Lugol's solution and chitin was formed, the area thus did not stain the purple-pink color of Lugol's solution.

It was previously found that chitinase secreted by *S. flaveus* was capable of utilizing the dry cells of *N. dimidiatum*, a causative agent of the brown spot disease in dragon fruit plants (Phan et al., 2023). *S. flaveus* was also reported to produce the manumycin-type antibiotic called SW-B, which was able to inhibit the growth of *Fusarium oxysporum* f.sp. *cucumerinum* although at a much lower level in comparison to many other plant pathogenic fungi (Hwang et al., 1996). *F. oxysporum* is also a causative agent of dry rot disease in potato tubers (Akosah et al., 2021). *F. oxysporum* is closely related to *F. solani*. Therefore, it is likely that SW-B could also inhibit the growth of *F. solani* DL. As presented above, it has been shown that the extracellular metabolites of *S. flaveus* were capable of inhibiting the growth of *F. solani* DL (Figure 3c), and the chitinase produced by *S. flaveus* was extracellular (Figure 3d). Also, culture media containing different ingredients resulted in different levels of chitinase (Figure 4a and b). To see if the extracellular chitinase produced by *S. flaveus* would be affected by the *F. solani* DL cell walls which are largely composed of chitin, the *S. flaveus* culture broth was supplemented with the *F. solani* DL dry cells and the level of chitinase production was investigated. Colloidal chitin was also added to the broth to ensure the detection of chitinase. Chitinase acted on colloidal chitin could release N-acetyl- $\beta$ -D-glucosamine. This reducing sugar was then quantified by reacting with the DNS reagent, yielding 3-amino-5-nitrosalicylic acid which could absorb visible light at 540 nm. Figure 4c shows that the chitinase production was significantly affected by the ratio between the colloidal chitin and fungal cell powder ( $p = 0.0421 \times 10^{-43}$ ). It was also significantly affected by the culture time ( $p = 0.0117 \times 10^{-35}$ ). Chitinase production varied during the culture time was also reported previously (Asif et al., 2019). Comparisons of the means of the  $OD_{540\text{ nm}}$  values in each pair of the ratios between the colloidal chitin and fungal cell powder showed that 5 out of 6 pairwise combinations (except the "1:2 versus 1:3" pair) having their means significantly different (p-values ranged from  $0.0148 \times 10^{-12}$  to  $0.0284 \times 10^{-6}$ ), and in each pair of the culture time showed that 26 out of 28 pairwise combinations (except the "48 h versus 84 h" and "60 h versus 72 h" pairs) having their means significantly different (p-values ranged from  $0.0197 \times 10^{-12}$  to 0.0367). The finding shows that culture broths with different concentrations of the colloidal chitin and fungal dry cells indeed resulted in different amounts of chitinase. Besides the ability to produce SW-B, the utilization of the fungal dry cells by *S. flaveus* makes this actinomycete a promising biocontrol agent for *F. solani* DL. Chitinase produced by this actinomycete will be an antifungal metabolite that needs more investigation with the fungal dry cells for increasing its concentration in the *S. flaveus* biocontrol preparation.

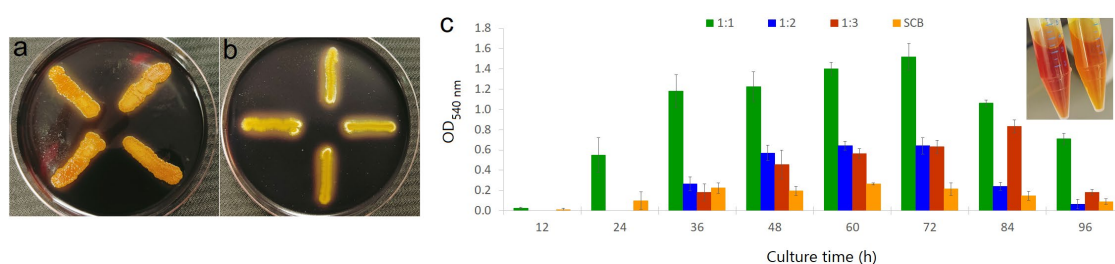


Figure 4: Actinomycete's hyphal mats on (a) the colloidal chitin agar and (b) the chitin casein agar; (c) Changes in chitinase production by *S. flaveus* in culture broth supplemented with colloidal chitin and fungal cell powder at different ratios. 1:1, 1:2 and 1:3 denote the ratios between colloidal chitin and fungal cell powder supplemented into the culture broths. Inset: a sample (left) containing 3-amino-5-nitrosalicylic acid and a blank (right) containing only DNS

## 4. Conclusions

The *F. solani* DL which caused the diseased dry rot potato in Da Lat City, a place where the geographical and natural conditions are very suitable for growing potatoes in Vietnam, was successfully isolated. It was sensitive

to *S. flaveus* which produced extracellular chitinase whose concentration in the liquid culture was affected by the supplementation of colloidal chitin and *F. solani* DL cell powder. Therefore, the *F. solani* DL mycelial cells will be an indispensable factor in the process of optimizing chitinase concentration in future *S. flaveus* preparation used for prevention and treatment of the potato dry rot disease.

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