Nguyen et al | Article 10221
J Pure Appl Microbiol. 2025;19(1):542-557. doi: 10.22207/JPAM.19.1.44
Received: 08 January 2025 | Accepted: 05 February 2025
Published Online: 28 February 2025

P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Biological Control of *Streptomyces murinus* against *Colletotrichum* causing Anthracnose Disease on Tomato Fruits

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Abstract

***Streptomyces* is a genus recognized for combating many plant pathogens, including those affecting post-harvest fruits. This study aimed to identify the *Colletotrichum* fungus responsible for anthracnose in tomatoes and evaluate the antifungal efficacy of *Streptomyces murinus* NARZ. The *Colletotrichum* isolate was identified as *C. scovillei* C3 through morphological characteristics, ITS region sequencing, and phylogenetic analysis. Culture filtrates (CF) of *S. murinus* NARZ at concentrations from 0% to 50% (in 10% intervals) were tested for antifungal activity against *C. scovillei* C3. The Percentage Inhibition of Radial Growth, calculated using colony diameters, showed that a 30.40% CF concentration (EC50) inhibited 50% of *C. scovillei* C3 growth on PDA plates. The CF exhibited heat stability, with PIRG values ranging from 62.93% to 65.35% across temperatures of 30 °C to 90 °C. *In vivo* trials involved treating tomatoes with CF using pre-treatment (spraying 24 hours before inoculation) or post-treatment (spraying 24 hours after inoculation). After seven days, pre-treated tomatoes with 50% CF showed a disease incidence of 83.33%, while all other treatments had a 100% incidence. Lesion diameters in the 50% CF treatment were significantly smaller ($p < 0.05$) compared to lower CF concentrations and showed results similar to the Chlorothalonil treatment. Pre-treatment was more effective than post-treatment, with lesion diameters of 5.40 mm and 8.73 mm, respectively. PCR analysis confirmed that *S. murinus* NARZ produced antifungal compounds via PKS-I, PKS-II, and NRPS gene clusters. These findings suggest that *S. murinus* NARZ could be an effective alternative to chemical fungicides for managing tomato anthracnose caused by *C. scovillei*.**

Keywords: Antifungal Activities, Anthracnose, *C. scovillei*, *Streptomyces murinus*, Tomato Fruit

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Citation: Nguyen HT, Le TL, Nguyen TDH, Truong TTT, Nguyen TTT. Biological Control of *Streptomyces murinus* against *Colletotrichum* Causing Anthracnose Disease on Tomato Fruits. *J Pure Appl Microbiol.* 2025;19(1):542-557. doi: 10.22207/JPAM.19.1.44

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is an important crop cultivated in many countries, including Vietnam. It is rich in various antioxidant compounds such as carotenoids, particularly lycopene, ascorbic acid, vitamin E, and phenolic compounds, especially flavonoids.[1] However, tomatoes are susceptible to various diseases, including severe fungal infections that threaten global tomato production, of which anthracnose is the most notable.[2]

Anthracnose, caused by *Colletotrichum* species, is a significant disease affecting tomatoes worldwide, leading to post-harvest fruit decay and severely reducing marketability.[3] Numerous *Colletotrichum* species are responsible for tomato anthracnose, including *C. gloeosporioides,*[4] *C. gossypii,*[5] *C. acutatum,*[6] *C. truncatum,*[7] *C. scovillei,*[8] *C. coccodes,*[9] and *C. nymphaeae,*[10] are emerging pathogens causing significant annual yield losses.

Infections by *Colletotrichum* species in tomato fruit transition from quiescent to active infection as the fruit ripens, resulting in anthracnose in mature tomatoes. When the fruit ripens, *Colletotrichum* spp. penetrate the pericarp, causing circular depressions that evolve into necrotrophic lesions,[11] displaying a dark, sunken, and water-soaked appearance.[7] Orange conidial masses appeared on the lesions, sometimes in concentric rings, and black acervuli formed just beneath the fruit skin.[6] The anthracnose infection shortens the shelf life of tomatoes[12] and reduces crop yields, resulting in a 10-30% loss of total crop production.[13] These losses diminish farmers' income and disrupt food supply chains, putting additional pressure on global agricultural markets.[2]

Chemical pesticides remain the most common method for controlling post-harvest diseases in fruits and vegetables. However, their improper use threatens human health, fosters pathogen resistance, and contributes to environmental pollution.[14-16] In response, biological control using antagonistic microorganisms such as bacteria, yeasts, molds, and actinomycetes has gained traction due to growing concerns about sustainability.[2] Biocontrol agents (BCAs) offer an eco-friendly alternative, with *Streptomyces* standing out for its ability to produce fungicides, antibiotics, and hydrolytic enzymes that inhibit phytopathogens. Unlike synthetic pesticides, *Streptomyces* suppresses plant diseases naturally, enhances immunity, promotes growth, and improves soil health. With stricter pesticide regulations and a shift toward sustainable agriculture, microbial biocontrol provides a safer, more effective solution.[17]

Several studies have utilized biological compounds derived from *Streptomyces* to inhibit pathogenic fungi and extend the shelf life of post-harvest fruits and vegetables.[18,19] *Streptomyces* sp. A1022 inhibited the growth of *C. gloeosporioides*, which causes anthracnose in pepper and cherry tomatoes.[16] *S. tuirus* AR26 was effective against infection and growth of *C. scovillei, C. truncatum,* and *F. oxysporum* on chili fruits.[20] *S. ahygroscopicus* was used for the post-harvest preservation of wax apples and guavas, enhancing their shelf life by delaying quality decline and reducing decay incidences.[21] *S. murinus* JKTJ-3 significantly inhibited *Pythium aphanidermatum*, which causes damping-off in watermelon, and exhibited broad antifungal activity against various fungi, including *Rhizoctonia solani, Colletotrichum gloeosporioides, Botrytis cinerea, Stagonosporopsis cucurbitacearum, Verticillium dahlia, Phomopsis vexans, Fusarium oxysporum* f.sp. *hiveum, Leptosphaeria biglobosa, Phomopsis asparagi, Fusarium solani, Colletotrichum capsic*, and *Sclerotiorum*.[19] More recently, *S. distatochromogenes* XT34 effectively controlled banana anthracnose caused by *C. musae* and maintained fruit quality during storage.[22] Metabolites from *S. murinus* THV12 inhibited effectively against *Candida albicans,*[23] while *S. murinus* SPL-1 showed effectiveness against *Trichomonas vaginali*.[24] The antifungal effects of *Streptomyces* spp. are believed to result from the presence of biosynthetic gene clusters (BGCs), which are responsible for antibiotics production, such as non-ribosomal peptide synthetase (NRPS), polyketide synthase (PKS), and other ketide synthases.[25]

Previously, we have demonstrated that *Streptomyces murinus* NARZ is a potential biological control agent against *Penicillium digitatum* and *Penicillium italicum* on oranges,[26] *C. truncatum* causing anthracnose on dragon fruits.[27] This study aimed to isolate and identify the fungus causing anthracnose in tomatoes and

evaluate the effects of culture filtrate from *S. murinus* NARZ in controlling *C. scovillei* growth on PDA plates and anthracnose disease on tomatoes. Furthermore, the putative genes encoding for its antibiotic production were also investigated.

MATERIALS AND METHODS

Materials

Tomatoes with typical anthracnose lesions were collected from local markets in Hue City, Vietnam. The fruits were contained in plastic bags with detailed information (collected date, places) and transferred to the laboratory for *Colletotrichum* sp. isolation.

Fresh, healthy tomatoes were purchased from markets in Hue City, Vietnam, and tomatoes were purchased at pink ripeness (equivalent to 2/3 ripeness) at the ripening stage and transferred to the laboratory.

Streptomyces murinus NARZ strain (PQ680203) was deposited at the Faculty of Engineering and Technology Laboratory, University of Agriculture and Forestry, Hue University, Vietnam.

Methods

Isolation and identification of *Colletotrichum* sp. causing anthracnose on tomatoes

Colletotrichum sp. was isolated from anthracnose lesions on tomatoes according to the method of Shahriar *et al*.[7] The fruits were surface disinfected as follows: rinsed under running tap water, surface disinfected with 70% ethanol for 3 min, soaked in 1% sodium hypochlorite for 3 min, and finally washed three times with sterilized distilled water. From the edge of the developing lesion, 2 mm^2 tissues were cut using a sterilized knife, and each piece was placed onto the center of Petri plates containing PDA (Potato dextrose agar) medium. These plates were incubated at 25 ± 2 °C for seven days. The resulting fungi were purified by transferring hyphal tips on new PDA plates. Pure colonies were preliminarily examined for *Colletotrichum* species based on their colony and spore morphological characteristics described in previous studies.[28-30]

The suspected *Colletotrichum* sp. was sequenced based on the ITS region by the DNA sequencing company (Can Tho, Vietnam). The resulting sequence was compared with the databases on NCBI (National Center for Biotechnology Information) using BLASTn to determine its taxonomy based on similarity. A phylogenetic tree was built based on resulted sequences and reference strains deposited on GenBank using MEGA11[31] to identify species classification. The identified strain was artificially re-inoculated into healthy tomatoes to test its pathogenicity. Furthermore, the fungus was reisolated from the developing lesion to test its morphologies compared to the inoculated strain.[32]

Culture filtrate (CF) preparation

S. murinus NARZ was cultured in ISP4 medium (International Streptomyces Project 4) on a shaker with 180 rpm at a temperature of 28 ± 2 °C for seven days. The culture was centrifuged at 10,000 rpm for 10 minutes at 4 °C. The supernatant was collected and passed through a 0.2 µm syringe Millipore filter. The obtained cell-free filtrate was used for antifungal examinations.[33]

Antifungal effect of CF from *S. murinus* NARZ against *C. scovillei* C3 growth

The CF was added to the PDA plates at 10%, 20%, 30%, 40% and 50% (v/v) concentrations. Plates without CF addition and plates containing nystatin (100 µg/mL) were negative and positive controls, respectively. From seven-day *C. scovillei* C3 plates, 2 mm^2 pieces were removed from the colony edge using a sterilized knife and placed onto the center of the prepared plates. These plates were incubated at 28 ± 2 °C, and colony diameters were recorded for seven days. Each concentration was repeated thrice. The inhibitory effect of the CF against *C. scovillei* C3 growth was assessed based on the Percentage Inhibition of Radial Growth (PIRG%). The PIRG was calculated as PIRG = (R_1 - R_2) * 100/R_1 whereas R_1 and R_2 were the diameter of the colonies in the control and tested plates, respectively (mm).[34] The concentration inhibited 50% of the growth of *C. scovillei* C3 (EC50 value) calculated based on the logarithmic regression equation generated from the PIRGs and CF concentrations graph.[35]

Heat stability of CF from *Streptomyces murinus* NARZ against *C. scovillei*

The CF of *S. murinus* NARZ was heated at temperature ranges of 30 °C, 50 °C, 70 °C, and 90 °C for 30 minutes and then separately added to the PDA medium at a concentration of 50%. A 2 mm^2 piece of seven-day-old *C. scovillei* C3 was placed at the center of the plates. The fungal diameters were recorded after 7 days incubated at 28 ± 2 °C. The PIRG (%) was calculated using the above equation. Plates without CF addition were controls.[33] Each heat treatment was repeated three times.

***C. scovillei* C3 spore suspension preparation and artificial inoculation**

The spore suspension was prepared from plates containing *C. scovillei* C3 after seven days incubated at 28 ± 2 °C on PDA medium plates. These plates were flooded with sterilized distilled water, and the colony's surface was scratched using an inoculating loop. The spore suspension was collected through a sterilized four-layer cheesecloth. Spore concentration was adjusted to 10^5 spore/µL using a hemocytometer for artificial inoculation.[36]

Healthy tomatoes were surface cleaned under running tap water; then they were dipped in ethanol 70% for 10 min and then in sodium hypochlorite 2% for 3 min. Finally, they were washed with sterilized distilled water three times and dried using sterilized tissue. On the equator of each fruit, a wound (2 x 2 x 2 mm in width, length, and depth) was created using a sterilized knife. Each tomato was artificially inoculated with *C. scovillei* by pipetting 10 µL prepared spore suspension onto the wounds.[37]

Effect of the CF on anthracnose development in tomatoes

Two treatment methods were conducted to evaluate the effect of the CF collected from *S. murinus* NARZ against anthracnose caused by *C. scovillei* C3 on tomatoes. The first treatment was tomato fruits, which were inoculated with 10 µL *C. scovillei* C3 prepared spore suspension for 24 hours and then sprayed with the CF using an aerosol sprayer (post-treatment). The second was tomato fruits were sprayed with the CF for 24 hours and inoculated with 10 µL *C. scovillei* C3 spore suspension afterward (pre-treatment). The CF concentrations in both treatments were 10%, 20%, 30%, 40%, and 50% (v/v). The two treatments were conducted in parallel and repeated three times for each. Sterilized distilled water and Chlorothalonil 0.026% were used as negative and positive controls, respectively. The control fruits were treated in the same way as described.[38]

All treated fruits were individually placed in plastic boxes (173 x 118 x 70 mm) disinfected with ethanol 70% and containing moistened sterilized tissue for seven days at 28 ± 2 °C. Disease incidence and lesion diameters were recorded every two days. Disease incidence was calculated using the formula DI = number of infected wounds * 100/total number of wounds. The PIRGs (%) were calculated using the formula PIRG = $(R_1 - R_2) * 100/R_1$, whereas R_1 and R_2 were the diameter of lesions in the control and tested fruits, respectively (mm).[38]

Determination of genes encoding for antibiotics production of *S. murinus* NARZ

Degenerate primers were designed to recognize conserved regions in PKS-I ketosynthase (KS), NRPS adenylation (AD), PKS-II ketosynthase

Table 1. Primer sequences used to amplify putative genes emcpdomg for antibiotic production

Genes	Length (bp)	Primers	Primer sequences (5' to 3')
Type I polyketidesynthase β-ketoacyl synthase (KS) domain fragments	670	KS-F	CCS CAG SAG CGCSTS YTS CTS GA
		KS-R	GTS CCS GTS CCGTGS GYS TCS A
Type II polyketide synthases KSα and KSβ domain fragments	800-900	KSα	TSG RCT ACR TCAACG GSC ACG G
		KSβ	TAC SAG TCS WTCGCC TGG TTC
Type II polyketide synthases KSα domain fragments	613	KS1-F	TSG CST GCT TGGAYG CSA TC
		KS1-R	TGG AAN CCG CCGAAB CCT CT
NRPS Adenylation domain (AD) fragments	700	A3F	GCS TAC SYS ATSTAC ACS TCS GG
		A7R	SAS GTC VCC SGTSCG GTA S

alpha (KSα), and ketosynthase beta (KSβ) that are responsible for antibiotic biosynthetic of *S. murinus* NARZ strains (Table 1).

The 25 µL reaction mixture for PCR contained 12.5 µL Master Mix (2X) (Bioline, England), 2 µL forward primer (10 µM), 2 µL reverse primer (10 µM), 7.5 µL DEPC water, and 1 µL 140 ng DNA template. Reaction mixtures were prepared for each DNA sample. The primer pairs KS-F and KS-R were used to amplify the genes that synthesize the polyketide synthase type I β-ketoacyl synthase (KS) region fragments. The PCR cycle consisted of an initial denaturation at 95 °C for 15 min, one cycle of 95 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min, followed by 35 cycles of 95 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min, with a final extension of 10 min at 72 °C. The PKS-II ketoacyl synthase alpha (KSα) and ketoacyl synthase beta (KSβ) regions were amplified using two sets of primers: KSα and KSβ and KS1-F and KS1-R. The PCR cycle was as follows: initial denaturation at 95 °C for 5 min; 40 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min; and a final extension for 10 min at 72 °C. Meanwhile, to amplify AD gene fragments, A3F and A7R primer set was used. PCR conditions included an initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 59 °C for 1.5 min, and 72 °C for 1 min, with a final extension for 10 min at 72 °C. PCR amplicons were analyzed using a 2% (w/v) agarose gel to determine the presence of the target genes.[25]

Statistical analysis

All experiments were performed in triplicates. Statistical analysis was performed using the SPSS statistics program 20 (IBM, USA) based on average values of three replicates. One-way ANOVA (Duncan's Multiple Range Test) was used to evaluate the differences in the PIRG% and lesion diameters at various concentrations of CF. The paired-sample t-test was used to compare the efficiency of the two treatments of CF on anthracnose lesion diameters in tomatoes.

RESULTS

Fungal isolation and identification

An isolate named C3 was purified from tomato fruits, which showed anthracnose symptoms, and its morphologies were examined. The mycelium was grayish-white to grayish-green, creating concentric circles. The mycelium was thick and fluffy, with abundant small orange and slimy spores. The back of the plate was light orange, and black spots formed on it. The diameter of the colony after 7 days was 47 mm. The spores (40 x magnification) were transparent and cylindrical, with blunt ends and straight shapes, showing oil droplets inside (Figure 1).

Our observation was similar to the previous description of *Colletotrichum* species. According to the description of Damm *et al*. an isolate with cottony hyphae, pale white to pale gray, with an olive gray center, and cylindrical transparent spores with a slightly pointed tip and a rounded tip belonged to the acutatum complex.[29] Furthermore, Kanto *et al*. also described the colony on PDA of *C. scovillei* as gray or pale gray to pale orange, sometimes with black spots on the back. However, traditional taxonomy based on morphological characteristics is unreliable because

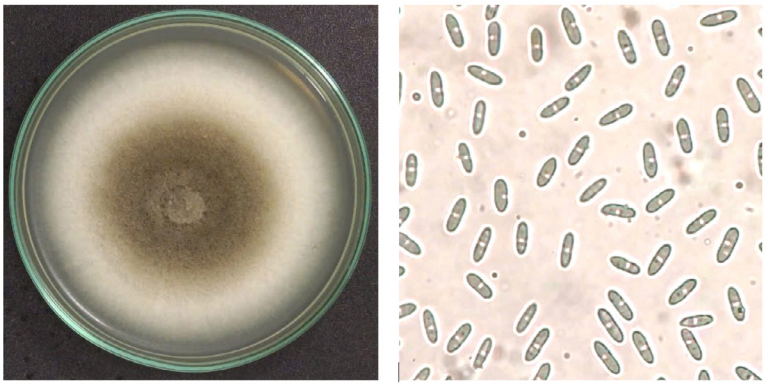

Figure 1. Conoly of *C. scovillei* C3 on PDA plates after seven days (left) and conidia (right).

Colletotrichum species are significantly affected by cultural conditions such as light, temperature, etc.[39] Therefore, molecular analysis based on the ITS region of isolate C3 was conducted to identify its taxonomy. The BLASTn result exhibited isolate C3 was 99.11% similar to *C. scovillei* C212 (MK327142.1). Furthermore, phylogenetic analysis indicated that isolate C3 had the nearest relationship with *C. scovillei* LD242 (LC488852.1) (Figure 2).

Therefore, it can be concluded that isolate C3 was *C. scovillei* and named *C. scovillei* C3. *C. scovillei* C3 was re-infected in tomato fruits to test its pathogenicity. The inoculated tomatoes showed typical symptoms of anthracnose. The lesions were circular, slightly sunken, and waterlogged, with orange sclerotia at the inoculated site, white mycelium formed around them and spread over the entire fruit surface. These symptoms were similar to the anthracnose lesions on the initially infected tomatoes (Figure 3). Furthermore, fungi re-isolated from the lesion had identical morphological characteristics with *C. scovillei* C3.

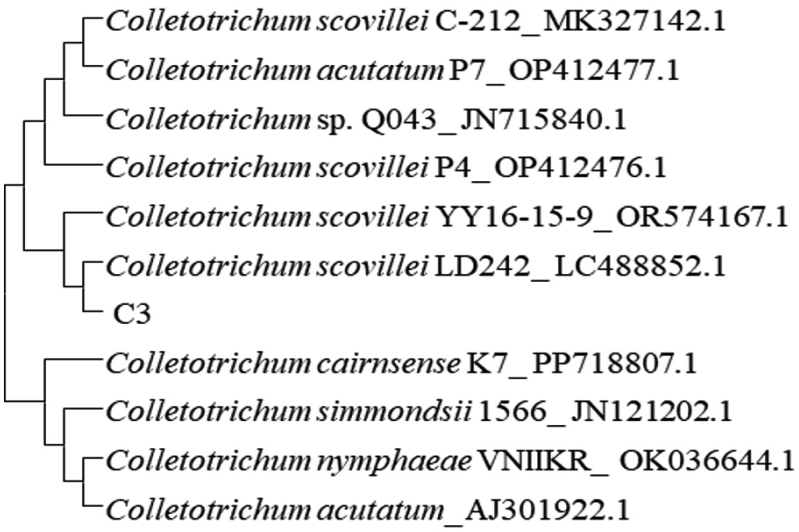

Figure 2. A phylogenetic tree was built using ITS region sequences of isolate C3 and reference sequences downloaded from GenBank

Figure 3. Anthracnose symptoms on tomato re-infected with *C. scovillei* C3

Effect of the CF against the growth of *C. scovillei* C3

Colony diameters of *C. scovillei* C3 were significantly smaller in the plates containing the CF of *S. murinus* NARZ than those of control plates without CF addition. The PIRGs (%) increased as the CF concentration was raised (Figure 4), gradually rising from 14.23% to 65.52%. The concentration inhibited 50% of the growth of *C. scovillei* C3 (EC_{50} value) was 30.40%, calculated based on a regression equation y = 33.102; ln(x) - 63.022 (R^2 = 0.9907). Nystatin (100 µg/mL) showed 53.8% PIRG against *C. scovillei* C3, lower than the efficiency of 40% CF, with 61.99% PIRG. The presence of CF not

only reduced the colony diameters of *C. scovillei* C3 but also altered its morphology. The colonies grew slower, and mycelia shrank, forming an orange layer in the center (Figure 5).

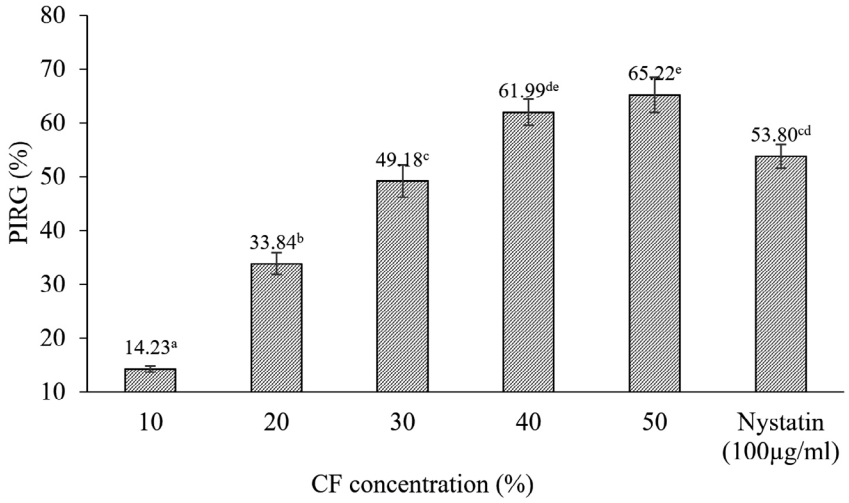

Figure 4. The PIRG (%) of CF from *S. murinus* NARZ against *C. scovillei* C3 after seven days

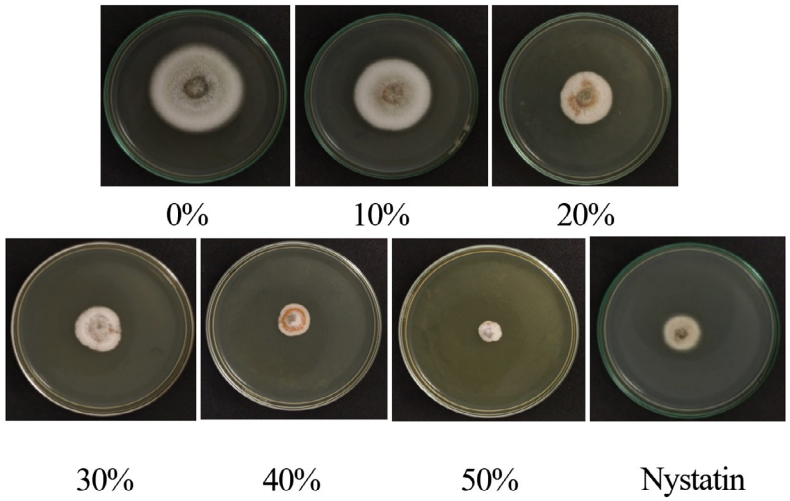

Figure 5. Colony morphologies of *C. scovillei* C3 on PDA medium with various concentrations of CF from *S. murinus* NARZ and controls after seven days

Heat stability of the CF against *C. scovillei* C3

The efficiency of the CF from *S. murinus* NARZ against *C. scovillei* C3 remained highly stable when subjected to heat treatment, with PIRGs fluctuating between 62.93% and 65.35%. After being treated at 90 °C for 15 minutes, the CF's efficiency against *C. scovillei* C3 was 62.93%, showing a 2.45% decrease compared to the 30 °C treatment. However, statistical analysis revealed no significant differences in PIRGs across all treatments ($p > 0.05$) (Figures 6 and 7).

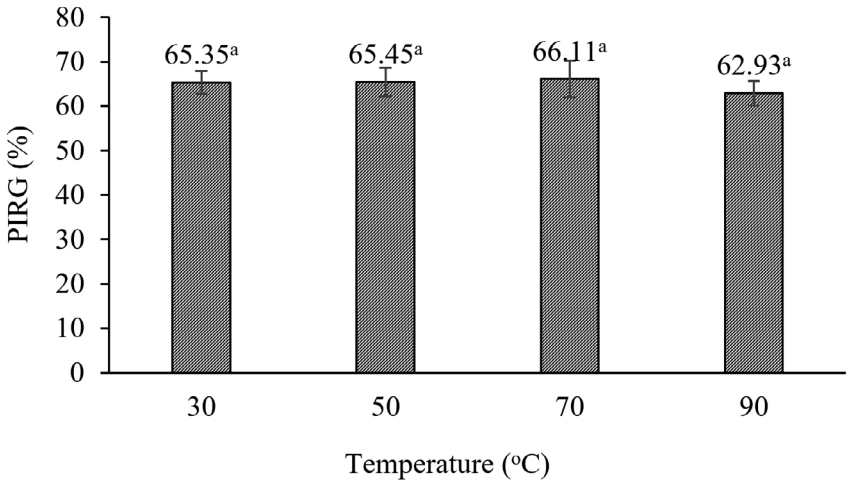

Figure 6. Effect of heat treatment on antifungal activities of CF from *S. murinus* NARZ against *C. scovillei* C3

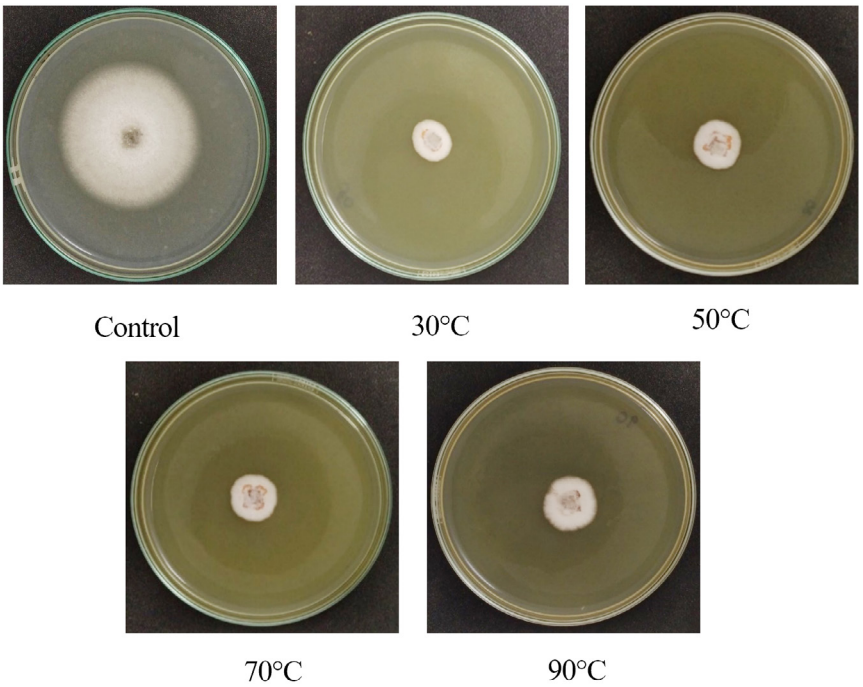

Figure 7. Colony diameters of *C. scovillei* C3 after 7 days on PDA medium with 50% CF treated at various temperatures

Effect of the CF from *S. murinus* NARZ against anthracnose disease caused by *C. scovillei* C3 on tomatoes

Table 2 indicates the positive efficacy of the CF from *S. murinus* NARZ against anthracnose disease caused by *C. scovillei* C3 on tomatoes under two treatments. However, the two treatments differed in disease incidences and lesion diameters (Tables 2 and 3).

After three days of incubation, all post-treated tomatoes exhibited anthracnose symptoms, resulting in a 100% disease incidence. In contrast, pre-treated tomatoes sprayed with over 20% CF before artificial inoculation showed no signs of anthracnose. Only the tomatoes treated with 10% CF exhibited a disease incidence of 66.67%. After five days, lesions developed at all inoculated wounds on tomatoes treated with 20%,

30%, and 40% CF, while those treated with 50% CF and Chlorothalonil (0.026%) showed disease incidences of 66.67% and 83.33%, respectively. Notably, after seven days, tomatoes treated with 50% CF had a disease incidence of 83.33%, while all other treatments reached 100%.

The CF treatments helped reduce tomato lesion diameters in both methods (Table 3). After three days of incubation, the lesion diameters reduced from 12.23 mm to 3.40 mm with 0% (control) to 50% CF in the post-treatment method. Lesions on tomatoes treated with 10% CF in pre-treatment were significantly larger than those in the post-treated tomatoes, 10.17 mm compared to 4.13 mm. After five days of treatment, there were no differences in lesion diameters in tomatoes treated with 10%, 20%, and 30% CF between the two treatments ($p \leq 0.05$). However, at 40% and 50% CF, lesions in pre-treated tomatoes were smaller than those of post-treated tomatoes. A significant difference in lesion diameters between the two treatments was observed only at a 50% CF concentration after seven days, with lesions measuring 5.40 mm in the pre-treatment and 8.73 mm in the post-treatment tomatoes ($p \leq 0.05$). Furthermore, the growth of lesion diameters in tomatoes treated with higher CF concentrations was slower than those treated with lower

Table 2. Anthracnose disease incidence (%) in tomatoes under the two treatments

CF (%)	Incubation time (days)					
	Pre-treatment			Post-treatment		
	3	5	7	3	5	7
0	100.00	100.00	100.00	100.00	100.00	100.00
10	66.67	100.00	100.00	100.00	100.00	100.00
20	-	100.00	100.00	100.00	100.00	100.00
30	-	100.00	100.00	100.00	100.00	100.00
40	-	100.00	100.00	100.00	100.00	100.00
50	-	66.67	83.33	100.00	100.00	100.00
Chlorothalonil (0.026%)	-	83.33	100.00	100.00	100.00	100.00

(-): no lesions

Table 3. Effect of CF from *S. murinus* NARZ on anthracnose lesion diameter (mm) on tomatoes

CFS (%)	Incubation time (days)					
	3		5		7	
	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment
0% (Control)	12.23 ± 0.68^{d}	11.53 ± 0.48^{b}	14.95 ± 3.92^{c}	16.50 ± 1.42^{d}	22.47 ± 1.91^{d}	23.87 ± 2.61^{e}
10%	10.17 ± 2.47*^{d}	4.13 ± 2.80^{a}	13.73 ± 2.11^{c}	11.63 ± 0.76^{c}	19.13 ± 1.52*^{d}	17.80 ± 0.62^{d}
20%	6.33 ± 1.53^{c}	-	10.17 ± 1.42^{bc}	9.37 ± 2.05^{bc}	16.77 ± 1.28^{c}	15.20 ± 1.39^{d}
30%	5.34 ± 0.58^{bc}	-	8.50 ± 2.14^{ab}	8.07 ± 1.12^{ab}	11.03 ± 1.68^{b}	10.40 ± 0.82^{c}
40%	4.67 ± 0.78^{bc}	-	6.90 ± 2.20*^{a}	5.50 ± 0.87^{ab}	9.80 ± 0.69^{b}	8.57 ± 1.60^{bc}
50%	3.40 ± 0.53^{b}	-	6.27 ± 3.84*^{a}	3.73 ± 0.32^{a}	8.73 ± 1.79*^{a}	5.40 ± 0.36^{a}
Chlorothalonil (0.026%)	-	-	5.10 ± 1.45^{a}	4.80 ± 1.69^{a}	7.63 ± 1.09^{a}	6.39 ± 1.25^{ab}

(-): no lesions.
In the same rows, the (*) following the SD values indicate a statistically significant difference at the significance level of $p \leq 0.05$.
In the same columns, different letters following the SD values indicate a statistically significant difference at the significance level of $p \leq 0.05$

concentrations during incubation time. At 10% CF in the post-treatment method, lesion diameters increased from 10.17 mm after three days to 19.13 mm after seven days, while tomatoes treated with 50% CF showed a slower increase from 3.40 mm after three days to 7.63 mm after seven days. Chlorothalonil (0.026%) demonstrated equivalent efficiency to 50% CF in preventing the progression of anthracnose.

Disease symptoms of post-treatment tomatoes were more severe than those in the pre-treatment at the same concentration of CF (Figure 8). The fruits were sunken, covered by the mycelium of *C. scovillei* C3 and orange conidia, with large cracks in fruits treated with 10%, 20%, and 30% CF concentrations and negative controls. Fruits treated with 50% CF had white mycelium with small lesions.

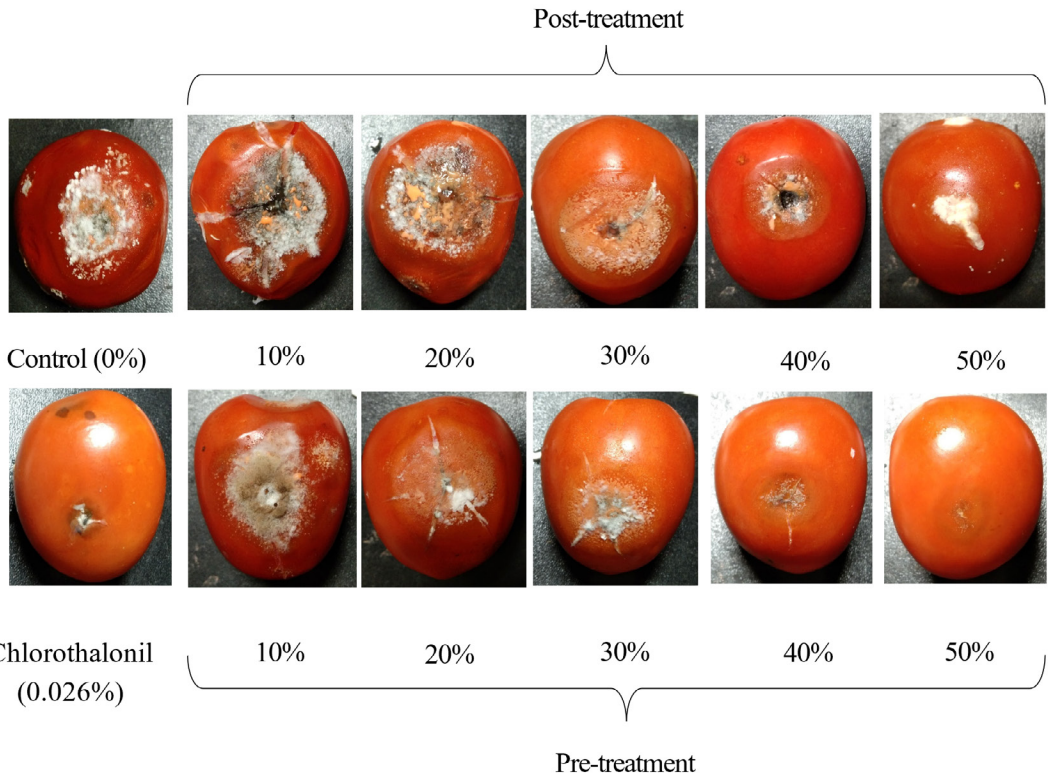

Figure 8. Anthracnose symptoms caused by *C. scovillei* C3 on tomatoes treated with CF from *S. murinus* NARZ at various concentrations in two treatments after seven days

Detection of genes encoding for antibiotic biosynthesis of *S. murinus* NARZ

Polyketide synthase type I (PKS-I), polyketide synthase type II (PKS-II), and non-ribosomal peptide synthase (NRPS) genes were identified through PCR analysis. The size of fragments varied from 700 to 850 bp (Figure 9), reaching the expected length when using their respective primer sets. The PKS-I gene was amplified using the KS-F and KS-R primer pair, with 700 to 800 bp length. The PKS-II gene was amplified using the KS1-F and KS1-R primer pair, yielding an approximate 800 bp fragment. The NRPS gene was amplified with the A3F and A7R primer pair, producing a fragment of approximately 800-850 bp. The KSα and KSβ primer pairs did not amplify any PKS-II gene fragments, suggesting either the absence of these PKS-II genes or the low specificity of the primers used. The presence of the polyketide synthase type I (PKS-I), polyketide synthase type II (PKS-II), and non-ribosomal peptide synthase (NRPS) genes contributed to explain the antibiotic biosynthesis of *S. murinus* NARZ.

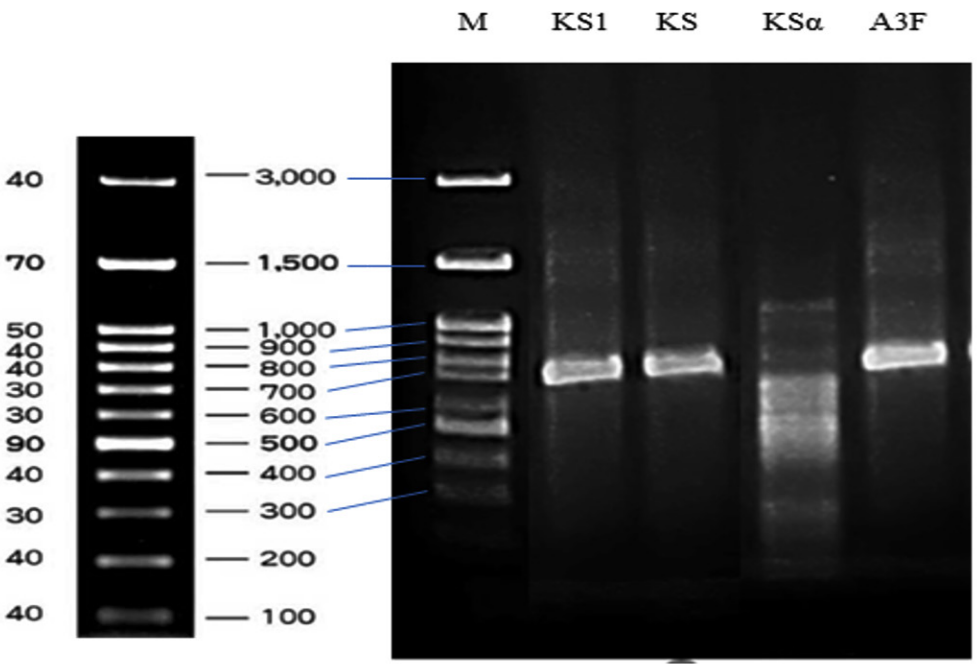

Figure 9. Agarose gel electrophoresis of PCR amplicons of type II polyketide synthases KSα domain fragments (Lane KS1), type I polyketide synthase β-ketoacyl synthase (KS) domain fragments (Lane KS), type II polyketide synthases KSα and KSβ domain fragments (Lane KSα), and non-ribosomal peptide synthase (NRPS) genes (Lane A3F)

DISCUSSION

It is essential to understand pathogen-causing diseases to have a proper controlling strategy. Therefore, the isolation of pathogen-causing anthracnose on tomatoes was necessary. Many *Colletotrichum* species have been recorded as causative agents in tomatoes, including *C. C. gloeosporioides,*[4] *C. gossypii,*[5] *C. acutatum,*[6] *C. truncatum,*[7] *C. scovillei,*[8] *C. coccodes,*[9] and *C. nymphaeae*.[10] However, anthracnose caused by *C. scovillei* on tomatoes has been rarely documented. This is the first report on *C. scovillei* causing anthracnose on tomatoes in Vietnam.

During the disease cycle, *C. scovillei* spores adhere to the surface and produce germ tubes (appressorium), specialized cells that invade host cells. After invading the epidermal cells, the fungus develops anthracnose lesions with typical sunken symptoms and pinkish-green acervuli containing large spores, essential for widespread infection. Polycyclic infection of the fungus contributes to significant disease during the growing season, causing severe economic losses.[40]

The antifungal activities of *S. murinus* species have been reported in previous studies, showing good effectiveness against *P. tabacinum*, which causes root rot disease in White Lupine,[41] *Pythium,* causing damping-off disease on watermelon,[19] *Candida albicans,*[23] and *Trichomonas vaginali*.[24] However, this study provides new insights into the antifungal activity of *S. murinus* NARZ, highlighting its beneficial effects in controlling tomato anthracnose caused by *C. scovillei* C3.

In our study, CF from *S. murinus* NARZ significantly reduced the growth of *C. scovillei* C3 in the PDA medium, with PIRG reaching up to 65.22% at 50% CF. CF of *S. katrae* had 95.00% inhibitory effect on the growth of *C. musae* mycelium on a PDA medium.[42] Meanwhile, *S. tuirus* showed antagonistic activity against chili rot pathogens, *C. scovillei*, *C. truncatum*, and *F. oxysporum*.[20] The authors explained the inhibitory activity

was often associated with disrupted mycelial supra-molecular organization. Culture extracts of *Streptomyces* spp. induced mycelial shriveling, exhibited fungal cell wall lytic activity, and inhibited spore growth.[1,43,44]

The antifungal properties of *S. murinus* have been attributed to the production of antibiotics such as Actinomycin X (Act-X), Actinomycin D (Act-D), Dactinomycin, Pentamycin, and other antifungal compounds.[19,23,24,45-47]

Although the use of Act-D in medicine is widely known, its use in agriculture is rarely documented. [35] Act-D synthesized by *Streptomyces* spp. showed effective in controlling plant pathogens and inhibiting the growth of *Fusarium* spp. causing wilt disease,[48] *B. cinerea,* which causes gray mold in tomatoes.[35] Additionally, Act-D produced by *Streptomyces* sp. Tc022 exhibited antifungal activity against *C. musae* with a minimum inhibitory concentration of 10 mg/ml.[49] Studies explored the mechanisms by which Actinomycin exerts its effects, such as damaging fungal cell membranes through a cleavage mechanism[50] and generating reactive oxygen species that harm fungal membranes.[51] Pentamycin, a polyene antifungal antibiotic produced by *Streptomyces* species, disrupts fungal cell membranes by binding to ergosterol, leading to cell death.[24]

On the other hand, the antifungal mechanism of *Streptomyces* spp. is believed to involve the production of cell-wall-degrading enzymes, such as β-1,3-glucanase and chitinase, which break down the cell walls of phytopathogenic fungi composed of chitins and glucans. Another secondary compound involved in this process is siderophores. *S. globisporus* F8 and *S. praecox* R7 have been shown to enhance lipoxygenase and phenylalanine ammonia-lyase activity in tomatoes, potentially triggering jasmonic acid and phenylpropanoid signaling pathways, thus activating a defense response in tomatoes against *R. solani*.[52]

Interestingly, our findings showed that the inhibitory effect of the culture filtrate (CF) from *S. murinus* NARZ against *C. scovillei* C3 was stable within a temperature range of 30-90 °C, the PIRGs remaining from 62.93% to 65.35%. Other studies also explained the inhibition of fungal growth by *Streptomyces* culture filtrate was associated with the presence of thermostable antifungal compounds rather than hydrolytic enzymes.[53,54] For example, the CF from the millet broth of *S. deccanensis* QY-3 demonstrated exceptional stability under thermal (20 °C-100 °C) and pH (2-10) conditions, effectively damaging the hyphae and inhibiting the germination of *C. gloeosporioides*.[42] Similarly, Wonglom *et al*. showed that the culture fluid of *S. angustmyceticus* NR8-2 displayed antifungal activity against *Colletotrichum* spp. and *Curvularia lunata*, maintaining stable antifungal properties even when exposed to temperatures of 28 °C, 50 °C, and 100 °C.[55] Our results suggested that the composition of the CF from *S. murinus* NARZ contained antibiotic compounds, which play a key role in antifungal activities, rather than enzymes that break down fungal cell membranes or other heat-sensitive compounds.

The application of CF obtained from *S. murinus* NARZ indicated the positive efficacy of preventing anthracnose disease caused by *C. scovillei* C3 in tomatoes. Pre-treatment tomatoes showed lower disease incidence than post-treatment tomatoes, with lesions measuring 5.40 mm in pre-treatment and 8.73 mm in post-treatment after seven days. At 50% concentration, CF was as effective as Chlorothalonil (100 µg/mL). These results were consistent with the report of Lian *et al*. Tomato samples 24 h pre-treated with 10 µL cell suspension of *S. pratensis* LMM15 (10^6 cells/mL) before artificial infection (pre-treatment) showed 76.64% antifungal efficacy against gray mold caused by *B. cinerea*. Meanwhile, procymidone pre-treated fruits had higher efficacy, reaching 83.18%. Compared with 24 h post-treated fruits, there were no differences in antifungal efficacy between cell suspension of *S. pratensis* LMM15 and Procymidone treatments, with 49.32% and 50.26%, respectively.[36] Thus, it can be concluded that fruits treated with antifungal compounds before the pathogen infection were more effective and efficient than treatments applied after the pathogen infection.

The antifungal mechanisms of *Streptomyces* spp. in inhibiting fungal growth on fruit include the suppression of mycelial growth and spore production, disruption of fungal cell walls and membranes, and interference with

mitochondrial function.[56] Spore germination is key in the early stages of fungal penetration and disease progression in fruit.[43] Prevention of spore adhesion results in fewer spore germination and forming an adsorbent layer on the plant surface, leading to a lower disease incidence. Furthermore, the enhanced inhibition observed with pre-treatment fruits can also be linked to the protease production of *Streptomyces* spp., which reduces sporulation, adhesion, and the formation of adsorbent layers. Protease preparations from *S. phaeourepureus* Expro 138 effectively reduced sporulation and prevented anthracnose disease in tomato plants.[57] Moreover, treatment with *Streptomyces* spp. can activate plant defense responses. Variations in antifungal activity may be influenced by factors such as the growth conditions of the microorganisms (e.g., carbon sources, nutrients, pH) and the inoculation method.[43]

Biosynthetic gene clusters (BGCs) are groups of genes responsible for producing bioactive compounds, such as antibacterial and antifungal agents.[24] *Streptomyces* species have demonstrated that their genomes possess abundant biosynthetic gene clusters (BGCs).[25] In our study, the presence of the polyketide synthase type I (PKS-I), polyketide synthase type II (PKS-II), and non-ribosomal peptide synthase (NRPS) genes in *S. murinus* NARZ contributed to explaining its antifungal action.

The *S. murinus* SPC1 strain, isolated from fox tail palm seeds, showed a potent antifungal effect against *Fusarium oxysporum* f. sp. *palmarum*, *Ganoderma zonatum*, and *Thielaviopsis paradoxa*. Genome analysis of *S. murinus* SPC1 revealed that this strain possesses a complete secondary metabolite cluster, 37 secondary metabolite BGCs, involved in producing the antifungal compound pentamycin.[58] Interestingly, a genome comparison study compared the genome of seven different *S. murinus* strains to the genome of *S. murinus* SPC1 revealed the presence of pentamycin-producing BGCs in all tested *S. murinus* strains.[24]

Antifungal compounds, including Actinomycin D, pentamycin, desferrioxamine E, and cinnabaramide A, were identified in the secondary metabolites of the *S. murinus* THV12 strain using MS/MS analysis. The *S. murinus* THV12 genome analysis revealed that the strain harbored 47 secondary metabolite biosynthetic gene clusters. Type 1 PKS gene clusters encode pentamycin production, while NRPS gene clusters are associated with actinomycin biosynthesis. Both type 1 PKS and NRPS gene clusters are responsible for cinnabaramide A synthesis, and siderophore gene clusters are related to desferrioxamin E secretion.[23]

The study by Tenebro *et al*. on the analysis of 19 *Streptomyces* species from marine sediments in the Philippines revealed variability among species regarding the presence of antibiotic biosynthesis genes. The results showed that all 19 *Streptomyces* species contained the NRPS gene based on amplifying the adenylation (AD) domain. Still, there were differences in the PKS-I and PKS-II genes across species. Most species contained the PKS-I gene, with only the *Streptomyces* sp. DSD3025 strain lacking PKS-I. Strains closely related to *S. mutabilis* did not have the conserved KS domain when amplified with the KS-F and KS-R primers. Diversity among species was particularly evident in the PKS-II domain. Only four species, *S. parvulus*, *S. griseorubens*, *S. carpaticus*, and *S. xiamenensis*, contained the KSα and KSβ domains of PKS-II, with a fragment size of 500-600 bp. Strains, such as *S. kunmingenesis*, *S. sedi*, and *Streptomyces* sp. DSD3025, lacked all PKS-II domains in their genome. The variability in PKS genes reflects differences in metabolite production, explaining species' observed variation in antibiotic activity.[25]

Based on the above analysis, we supposed that the *S. murinus* NARZ strain produced antifungal compounds, including pentamycin, actinomycin D, and other metabolites, through PKS-I, PKS-II, and NRPS gene clusters in its genome.

CONCLUSION

This study demonstrated that *S. murinus* NARZ effectively inhibited *C. scovillei* C3, the pathogen causing anthracnose in tomatoes. Its culture filtrate was highly thermostable, making it suitable for practical use. The application of CF on tomatoes infected with *C. scovillei* C3 suggests that antifungal treatments should be applied shortly after harvest to prevent pathogen invasion. This is

the first study to explore the biocontrol potential of *S. murinus* isolated from soil against *C. scovillei*, which was first recorded as a pathogen responsible for anthracnose on tomatoes in Vietnam.

ACKNOWLEDGMENTS

The authors would like to thank Prof. Dr. Markus Kalkum of the City of Hope, California, USA, for his assistance with this manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

HTN and TTTN designed the experiments. TLL, TDHN, and TTTT carried out the experiments and performed data analysis. HTN wrote the manuscript. TTTN revised the manuscript. All authors read and approved the final manuscript for publication.

FUNDING

This research was funded by the Hue University of Agriculture and Forestry, Hue University, under Project NCM.lHNL.2021-04. This research was also partly funded by the International Foundation for Science, under Project number I1-F-6276-1.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

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