Vietnam Journal of Biotechnology **23**(4): 555-566, 2025. DOI: 10.15625/vjbt-23226

ISOLATION AND CHARACTERIZATION OF ACTINOMYCETES AGAINST FUNGI CAUSING ANTHRACNOSE AND LEAF SPOT ON ROSE PLANTS

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Received: 28.07.2025
Accepted: 08.12.2025

ABSTRACT

Roses (*Rosa* spp., family Rosaceae) are one of the most iconic and beloved flowering plants worldwide. They serve not only as ornamental plants but also possess high economic value. However, rose production is currently threatened by various fungal diseases, including anthracnose, leaf spot, downy mildew, and powdery mildew, which reduce both productivity and quality. Therefore, the identification of actinomycete strains for the development of biological control agents is urgently needed. In this study, 17 strains of actinomycetes were isolated by using the gradient dilution and confrontation methods. Among them, the DT5 strain was selected for its strong antifungal activity, showing inhibition rates of 58.33% against anthracnose (R2 strain) and 52.17% against leaf spot (R3 strain) in roses. Colonies of the DT5 strain displayed a dry surface with concentric rings, initially white but gradually turning whitish-gray. Microscopic observation revealed elongated, branched hyphae and short, hook-shaped spore chains. Based on these morphological features, the DT5 strain was preliminarily classified as belonging to the genus *Streptomyces*. The DT5 strain was able to synthesize cellulase and effectively assimilate various carbon sources (such as glucose and sucrose) as well as nitrogen sources (including $NaNO_3$, beef extract, and peptone). Additionally, the DT5 strain grew well at temperatures ranging from 30-37°C and at pH 5.0-7.0. Under optimal conditions, after 5 days of culture at pH 6.0, the DT5 strain significantly enhanced its antifungal activity against both the R2 and R3 strains with inhibition zones of approximately 15 mm and 20 mm, respectively. Our results suggest that the DT5 strain has good potential as a biological control agent against fungal diseases. However, future *in vivo* experiments under greenhouse and field conditions are required to validate its antifungal efficacy. These efforts will guide the development of effective and sustainable biocontrol products not only for managing fungal diseases in roses but also for potential application in protecting other economically important crops.

Keywords: Actinomycetes, antifungal activity, anthracnose, leaf spot, pathogenic fungal strains, roses.

INTRODUCTION

Roses, often referred to as the queen of flowers, belong to the Rosaceae family and are among the most widely appreciated perennial flowering plants due to their aesthetic appeal. There are more than a hundred species and thousands of cultivars grown for various purposes. Most species of roses originated from regions such as Europe, the Americas, East Asia, or the Middle East (Beales, 1997; Joyaux, 2003). However, diseases represent one of the most significant threats to rose cultivation. Fungi are the primary pathogens responsible for causing a range of diseases across different rose-growing areas. Key fungal diseases include *Diplocarpon rosae* (black spot), *Cercosporarosicola* (Cercospora leaf spot), *Sphaerothecapannosa* (powdery mildew), *Peronospora sparsa* (downy mildew), and *Botrytis cinerea* (grey mold), which pose significant threats to crops worldwide (Pandya *et al.*, 2022). In addition, *Colletotrichum* sp. is known as one of the fungal genera capable of causing some common diseases on rose plants, such as anthracnose and leaf spots. In which, anthracnose has an infection rate of about 50-70% in the field according to a survey of 100 plants, while another study reported leaf spot severity ranging from 18.05% to 22.6% on sampled plants (Du *et al.*, 2023; Mohan *et al.*, 2024). Annually, the rose industry suffers about $10 million in losses from two rose diseases (leaf spot and rosette disease) (Gray, 2020). Although reports on yield and economic losses in roses are limited, diseases caused by *Colletotrichum sp.* are a major constraint on both quality and marketability. In roses, even a minor incidence of disease can lead to the rejection of entire batches of cut flowers due to strict aesthetic requirements (Pscheidt, 2025). Therefore, the economic impact of these diseases on rose production is considerable.

Currently, synthetic fungicides are the main method used to control these plant pathogenic fungi. However, these chemicals can be detrimental to humans, animals, and the environment, and they can lead to resistance in fungal populations. In recent decades, there has been a growing interest in using microorganisms, especially actinomycetes, as biological control agents against these pathogens, offering a viable alternative to synthetic fungicides. Actinomycetes are Gram-positive bacteria that thrive in a variety of environments with different moisture levels, pH, and temperature ranges (Jagannathan *et al.*, 2021). They have been isolated from terrestrial, marine, wetland, saline, and endophytic habitats and have shown promise in controlling plant pathogens biologically (Maua *et al.*, 2022; Tatar, 2021). Therefore, the aim of this study is to isolate, screen, and characterize antifungal actinomycetes from the root soil of rose gardens and to optimize specific culture conditions to enhance the antifungal activity of the selected actinomycete strain. The findings from this research could be vital in developing effective biological products for agriculture aimed at controlling fungal diseases in roses and potentially other crops.

MATERIALS AND METHODS

Materials

Colletotrichum sp. strain R2, causing anthracnose disease, and *Colletotrichum* sp. strain R3, causing leaf spot disease, on rose plants were stored at the Microbial Biotechnology department, Faculty of Biotechnology, Vietnam National University of Agriculture.

Rhizosphere soil samples were collected from rose gardens in Gia Lam commune, Hanoi; Phu Son commune, Hanoi; and Binh Luc commune, Ninh Binh. In each garden, soil samples were taken from five locations at a depth of 10-20 cm. The samples were placed in sterile plastic bags, sealed, and transported to the laboratory. The samples from each garden were then thoroughly mixed to obtain a composite sample, which was air-dried at room temperature, ground, and sieved through a 2 mm mesh to eliminate large debris. Finally, the samples were stored at 4°C before isolation was carried out (Antido & Climacosa, 2022).

Isolation of actinomycetes strains

Actinomycetes were isolated from soil samples collected using the gradient dilution separation method (Sadeghian *et al.*, 2016). The soil samples were ground into a powder and diluted at a ratio of 1:10 by adding 10 g of soil to 90 mL of sterile water, then shaken at 180 rpm for 30 minutes. After settling, the homogeneous solution was further diluted with sterile water to concentrations of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. A volume of 100 µL from each dilution was spread onto Petri plates containing Gause's N°. 1 agar medium.

The dishes were incubated at 30°C for 5-7 days, and the growth of actinomycetes was observed periodically. Subsequently, the actinomycete colonies were isolated, purified, and subcultured on Gause's N°. 1 agar plates for further experiments and storage.

Preliminary screening of antifungal actinomycetes against fungi R2 and R3 strains

Actinomycetes were tested for their antifungal activity against fungi R2 and R3 that cause diseases on roses using the confrontation method described by Jin *et al.* (2014), with some modifications. The isolated actinomycetes were streaked on two symmetrical points approximately 2-3 cm from the center of the Petri dish containing PDA medium and incubated at 30°C for 7 days. After incubation, a 5-day-old fungal disc (6 mm) was placed at the center of the PDA plate. The control plate was inoculated with only a fungal disc. The plates were then incubated at 30°C for 7 days, and the antifungal activity was assessed by determining the diameter of the inhibition zones (Jin *et al.*, 2014).

Morphological, physiological, and biological characterization of actinomycetes

The potent actinomycete strain selected from the primary screening was characterized using morphological, physiological, and biochemical methods. The selected strain was cultured in Gause's N°. 1 medium to assess its morphological characteristics through both macroscopic and microscopic techniques. Macroscopic characteristics included colony morphology in terms of the elevation form, aerial and substrate mycelium, and soluble pigments. For microscopic analysis, the strain was cultured on Gause's N°. 1 plates, and a sterilized coverslip was placed at a 45° angle using tweezers, then incubated at 30°C for 5-7 days. After incubation, the coverslip was carefully removed from the medium, and the morphological features of the aerial mycelium, substrate mycelium, and spores were analyzed using a light microscope (Duddu & Guntuku, 2016).

The physiological and biochemical characteristics of each isolated strain were

considered essential for identifying potent cultured strains. The growth of the strain was evaluated at different temperatures between 25 and 50°C and pH levels from 5.0 to 10 (Phan *et al.*, 2016; Shatri, 2024).

The ability to utilize carbon and nitrogen sources was assessed according to the methods of Canh *et al.* (2018), Nguyen *et al.* (2025), and Palla *et al.* (2018). The ability of actinomycete strains to use different sugars for energy and their growth was investigated by adding various sources of sugars (glucose, fructose, sucrose, and D-xylose) at a 1% concentration to ISP-9 medium (5.65 g/L K_2HPO_4, 2.38 g/L KH_2PO_4, 1.0 g/L $MgSO_4.7H_2O$, 2.64 g/L $(NH_4)_2SO_4$ and 15 g/L agar, pH 6.8-7.0), with growth measurements recorded after 5-7 days of incubation. Nitrogen sources, such as beef extract, KNO_3, $(NH_4)_2SO_4$, and peptone, were used to evaluate the isolated strain's ability to utilize them for energy production. The actinomycete strain was cultured on nitrate starch medium, where nitrogen sources could be replaced with $NaNO_3$ (Canh *et al.*, 2018; Nguyen *et al.*, 2025; Palla *et al.*, 2018).

The actinomycete strain was also cultured on ISP-6 (15 g/L peptone, 5 g/L proteose peptone, 1.0 g/L yeast extract, 0.5 g/L ferric ammonium citrate, 1.0 g/L $K_2HPO_4.3H_2O$, 0.08 g/L sodium thiosulfate, and 15 g/L agar, pH 7.2) to examine melanin pigment production, following the procedure described by Shirling and Gottlieb (1966). Melanin formation changed the medium's color from light yellow to brown and black (Shirling & Gottlieb, 1966).

Additionally, the activity of enzymes (amylase, protease, and cellulase) was determined using media with suitable substrates (starch, gelatin, and carboxymethyl cellulose). The actinomycete strain was cultured in Gause's N°. 1 at 30°C for 7 days. Following this, approximately 100 µL of the supernatant was added to the wells of appropriate media and incubated for 24 hours. Enzyme production was then assessed by the presence of a colorless halo around the wells when Lugol's iodine solution was added to the plates (Phan *et al.*, 2016; Topatan & Kati, 2022).

Optimization of culture time and pH conditions for maximum antifungal activity

The DT5 strain was grown in Gause's N°. 1 at different pH levels (5.0, 6.0, 7.0, 8.0, and 9.0), shaking at 180 rpm and 30°C. The supernatant was also tested for antifungal activity by using the agar disc diffusion method (Phan *et al.*, 2016; Vijayakumar *et al.*, 2012). Sterilized PGA medium plates were prepared, followed by the precise punching of a 0.5 cm diameter agar well in the center of each plate. Subsequently, 100 µL of the 7-day centrifuged actinomycete culture was added into the well. Pathogenic fungi (the R2 and R3 strains) were then dotted around the well, positioned 2-3 cm from its edge. All the plates were placed in a refrigerator at 4°C for 4 hours before being transferred to an incubator at 30°C, and the results were observed after 5 days of cultivation.

Similarly, the DT5 strain was cultured in Gause's N°. 1, shaking at 180 rpm and 30°C. After 3, 5, 7, and 9 days of culture, the supernatant was tested for antifungal activity by using the agar disc diffusion method (Vijayakumar *et al.*, 2012).

Statistical analysis

All experiments were performed in triplicate. Data were expressed as the mean ± standard

deviation. Pairwise comparisons were performed using Student's t-test, with statistical significance set at p-value < 0.05.

RESULTS AND DISCUSSION

Isolation and screening of antifungal actinomycetes against fungi causing diseases on roses

In this study, a total of 17 actinomycete strains were isolated from soil samples collected from rose gardens in Gia Lam commune, Hanoi; Phu Son commune, Hanoi; and Binh Luc commune, Ninh Binh. All isolates were screened for their resistance against the anthracnose-causing R2 and the leaf spot-causing R3 strains affecting rose plants by the co-culture method. The screening results indicated that, out of 17 isolated strains, three strains (DT2, DT4, and DT5) displayed good antifungal activity against the R2 strain, with antagonistic efficiencies of 47.92%, 41.67%, and 58.33%, respectively, while two strains (DT5 and DT9) showed good antifungal activity against the R3 strain, with antagonistic efficiencies of 52.17% and 43.48%, respectively. Among the screened actinomycete strains, the DT5 strain exhibited significant antifungal activity against both pathogenic fungi strains, R2 and R3, on rose plants, as evidenced by a strong inhibitory effect of the DT5 strain against the pathogenic fungal strains (Figure 1). To develop effective biological products, selecting actinomycete strains with good antagonism against many pathogenic fungi was being focused on. Therefore, the DT5 strain was chosen for further detailed studies on morphology, biochemistry, and other aspects.

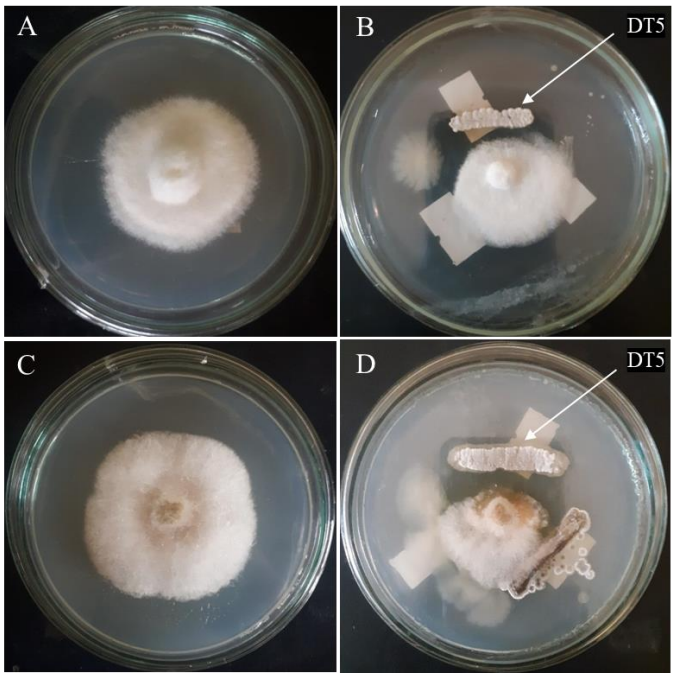

Figure 1. Antifungal effects of the actinomycetes DT5 strain against two fungal strains, R2 and R3, after 5 days of co-culture**.** (A) The R2 strain (control), (B) inhibition of the antifungal DT5 strain against the R2 strain, (C) the R3 strain (control), and (D) inhibition of the antifungal DT5 strain against the R3 strain.

Morphological, physiological, and biological characterization of the selected actinomycete DT5 strain

Morphological characteristics

The DT5 strain was cultivated on Gause's N°. 1 at 37°C and after 5 days of culture, the strain exhibited strong growth on the media (Figure 2A). The colonies of the DT5 strain had dry surfaces with concentric circles. The aerial mycelia and substrate mycelia were initially white, then gradually turned gray-white. The potent isolate DT5 was examined under a light microscope, and the result showed that the aerial hyphae were elongated and branched (Figure 2B). The hyphae then formed short, hook-shaped spore chains (Figure 2C). The characteristics of the colonies and mycelia of the actinomycete DT5 strain were similar to those previously described in Waksman's study on *Streptomyces* genus classification (Waksman, 1961). Many studies have also confirmed that various *Streptomyces* sp. strains possess the ability to antagonize *Colletotrichum* sp., responsible for anthracnose and leaf spots in plants. For example, the *S. griseorubiginosus* LJS06 strain effectively suppressed *C. orbiculare* COC3, causing anthracnose in cucumbers (Chai *et al.*, 2022). Similarly, *S. murinus* was shown to inhibit *Colletotrichum* sp., responsible for anthracnose in tomato fruits (Hien *et al.*, 2025). In addition, the *S. angustmyceticus* NR8-2 strain was found to control *Colletotrichum* sp., causing the leaf spot of *Brassica rapa* subsp. *pekinensis* (Wonglom *et al.*, 2019).

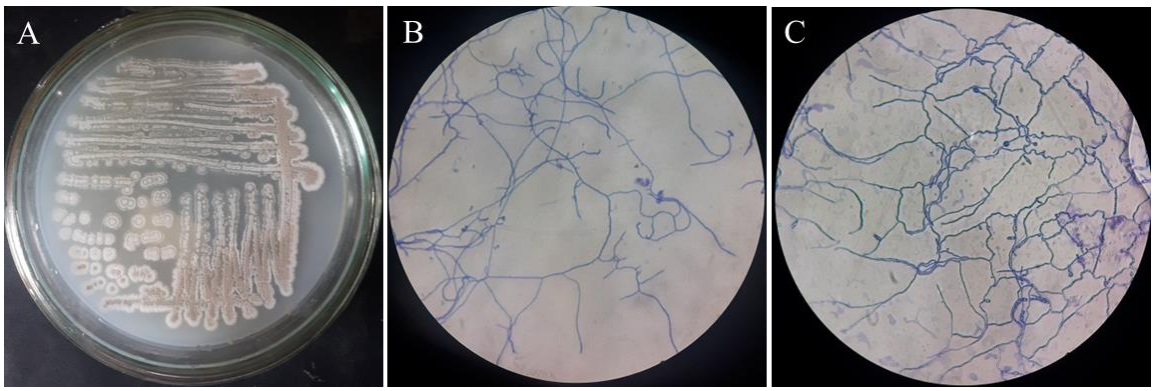

Figure 2. Morphological characterization of the DT5 strain. (A) Colony morphology of the DT5 strain, (B) mycelium morphology, and (C) spore chain morphology. Figures 2B and 2C were observed at 40X magnification.

Physiological and biochemical characteristics

The results regarding the physiological and biochemical characteristics indicated that the DT5 strain exhibited robust growth at temperatures between 30-37°C, moderate growth at 25°C and 45°C, but the growth was weak at 50°C (Table 1). The DT5 strain grew excellently at pH levels of 5.0-7.0, with good growth at pH 8.0, while its growth was weak at pH 9.0 and 10 (Table 1). The DT5 strain demonstrated a broad ability to utilize various carbon and nitrogen sources (Table 2). Specifically, this actinomycete strain could effectively assimilate sucrose and glucose as carbon sources and $NaNO_3$, beef extract, and peptone as nitrogen sources. Canh *et al.* (2018), Duddu and Guntuku (2016), and El-Naggar and El-Ewasy (2017)

also reported similar results that the selected actinomycete strain produced cellulase, widely utilized different carbon sources and nitrogen sources, and had an optimal growth temperature of 30°C and an optimal pH of 7.0 (Canh *et al.*, 2018; Duddu & Guntuku, 2016; El-Naggar & El-Ewasy, 2017). Biochemical analysis revealed that the DT5 strain could produce cellulase, indicated by a 2.4 cm clear zone on a cellulose containing agar plate, but not protease and amylase. Melanin pigment was also not detected on the ISP-6 medium.

Table 1. Effect of temperature and pH on the growth of the DT5 strain.

Temperature	**25°C**	**30°C**	**37°C**	**45°C**	**50°C**	
Growth of the actinomycete strain	++	+++	+++	++	+	
pH	5.0	6.0	7.0	8.0	9.0	10
Growth of the actinomycete strain	+++	+++	+++	++	+	+

Note: (+) weak growth; (++) moderate growth; (+++) good growth.

Table 2. Assimilation of different carbon and nitrogen sources of the DT5 strain.

Carbon sources	**Growth of the actinomycete strain**	**Nitrogen sources**	**Growth of the actinomycete strain**
Sucrose	+++	$NaNO_3$	+++
Fructose	+	Beef extract	+++
D-xylose	+	Peptone	+++
Glucose	+++	$(NH_4)_2SO_4$	++
		KNO_3	++

Note: (+) weak growth; (++) moderate growth; (+++) good growth.

Optimization of culture time and pH condition for maximum antifungal activity

The DT5 strain was inoculated in Gause's N°. 1 medium with shaking (180 rpm) at 30°C under different pH and incubation times. The extracts then were analyzed for antifungal activity by the well diffusion method. Statistical analysis using Student's t-test with a two-tailed *p*-value was conducted to evaluate the effects of pH and incubation time on the antifungal activity of DT5 culture supernatant against the R3 and R2 strains.

Effect of pH on antifungal production

When cultured in Gause's N°. 1 medium at different pH ranges, the level of antagonism exhibited by the DT5 strain varied (Figure 3). The DT5 strain also exhibited strong antagonism against the R3 strain in the pH range of 5.0-7.0, and at pH 6.0 the strain displayed the strongest ability to antagonize the R3 strain (about 13.5 mm) (Figure 3A). Similarly, the DT5 strain showed strong antagonism against the R2 strain in the pH range of 5.0-7.0, and the strongest inhibitory effect was also observed at pH 6.0 (about 17 mm) (Figure 3B). In both cases, the

antagonistic activity of the DT5 strain against the R2 and R3 strains gradually decreased in the pH range of 8.0-9.0. In addition, the results indicated that the inhibition zone diameters against R3 and R2 strains were significantly higher at pH 6 compared to pH 7.0, pH 8.0, and pH 9.0 ($p < 0.05$). However, no significant difference was observed between pH 6.0 and pH 5.0 for R3 strain inhibition ($p > 0.05$). These findings suggest that pH 6.0 is the optimal condition for culturing the DT5 strain to achieve maximum antifungal efficacy.

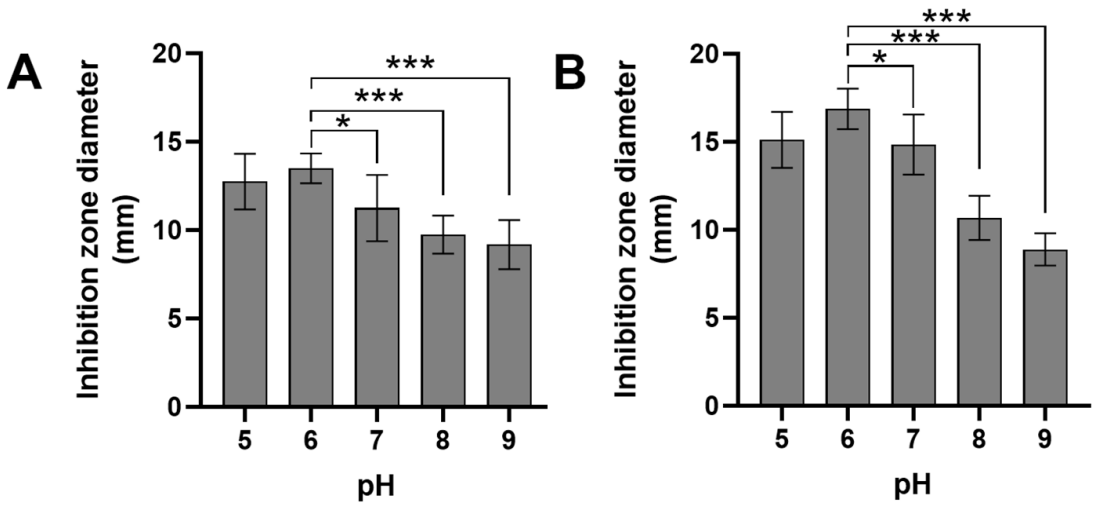

Figure 3. Effect of pH on the antifungal ability of the DT5 strain against fungal pathogens on rose plants. (A) Inhibition zone of the actinomycete DT5 strain against the R3 strain. (B) Inhibition zone of the actinomycete DT5 strain against the R2 strain (Mean ± SD, n = 3).

Effect of culture time on antifungal activity

The results showed that when the actinomycete DT5 strain was cultured in Gause's N°. 1 medium at different time intervals, its antifungal activity varied. The strain exhibited strong antifungal activity against the R3 strain during the culture period spanning from 3 to 7 days. The strongest antifungal effect of the DT5 strain against the R3 strain was observed after 5 days of culture (about 15 mm). The strain's antifungal activity exhibited a significant decrease after 9 days of culture (Figure 4A). Additionally, the inhibition zone diameter against the R3 strain was significantly larger after 5 days of incubation compared to 9 days ($p < 0.05$). Although there was no significant difference in the inhibition zone diameter of the DT5 culture after 5 days of incubation compared with 3 and 7 days, preliminary evaluation indicated that after 5 days, the DT5 strain may produce secondary metabolites capable of effectively inhibiting the R3 strain.

Likewise, the actinomycete DT5 strain also showed comparable antifungal activity against the R2 strain. During the culture period of 3-7 days, the DT5 strain exhibited strong antifungal properties towards the R2 strain, with the highest level of antagonism observed after 5 days of culture (about 20 mm). The antifungal activity of the DT5 strain clearly decreased after 9 days of culture (Figure 4B). Furthermore, the inhibition zone diameter after 5 days was significantly larger than after 3, 7, and 9 days

($p < 0.05$). These results indicated that a 5-day incubation period is optimal for enhancing the antifungal activity of DT5 culture supernatant. Thus, the antifungal activity against fungal pathogens on rose plants produced by the DT5 strain varied based on the pH of the medium and the culture time. In this study, when the DT5 strain was cultured in Gause's N°. 1 medium at a pH of 6.0 and at a temperature of 30°C for 5 days, the antifungal substances produced exhibited high inhibitory properties against the R2 and R3 strains. These results were also reported by Azish *et al.* (2020) in their study. The highest antifungal activity was observed in the medium at a pH of 7.0, maintained at 30°C for five days (Azish *et al.*, 2020). Similarly, Reddy *et al.* (2011) found that the maximum production of antifungal metabolites against *C. albicans* occurred at a pH of 7.5, a temperature of 32°C, and a culture duration of 5 days (Reddy *et al.*, 2011).

With its ability to grow well at 30-37°C and pH 5.0-7.0, and to effectively utilize diverse carbon and nitrogen sources, the DT5 strain clearly demonstrates strong environmental adaptability and enhances nutritional competition with fungi in general and *Colletotrichum* sp. in particular. Moreover, the DT5 strain also produces cellulase, an enzyme capable of decomposing cellulose and related polysaccharides in soil organic matter into oligosaccharides and glucose, which are readily available carbon sources for its growth (Behera *et al.*, 2017). Consequently, the DT5 strain can suppress the growth of *Colletotrichum* sp. by limiting its nutrient absorption and occupying ecological niches.

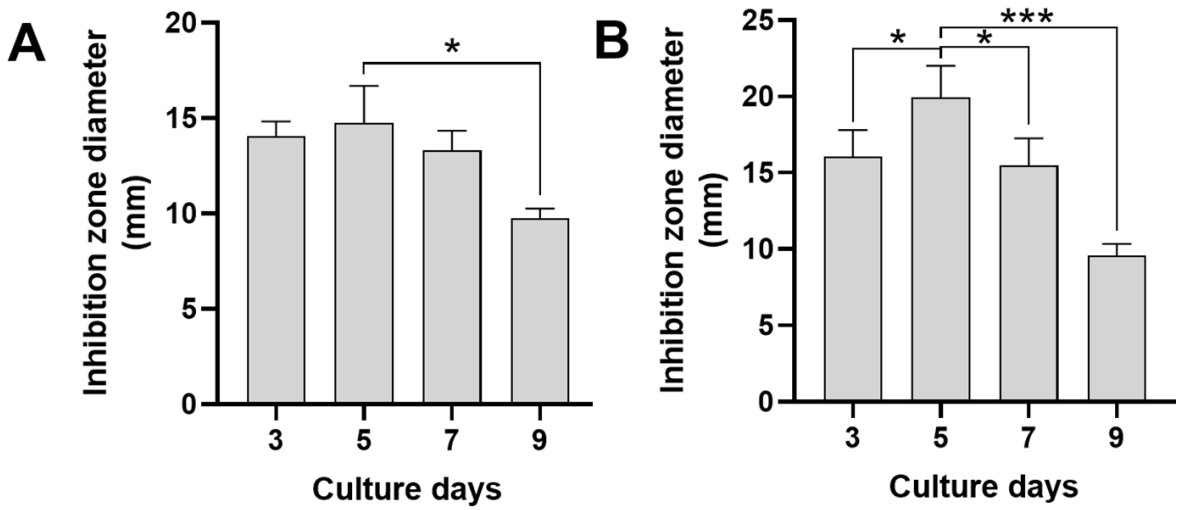

Figure 4. Effect of culture time on the antifungal ability of the DT5 strain against fungal pathogens on rose plants. (A) Inhibition zone of the actinomycete DT5 strain against the R3 strain. (B) Inhibition zone of the actinomycete DT5 strain against the R2 strain (Mean ± SD, n = 3).

Furthermore, the DT5 actinomycete belongs to *Streptomyces* sp., which is well known for producing secondary metabolites, including antibiotics, thereby enhancing its ability to antagonize *Colletotrichum* sp. (Carlos Daniel *et al.*, 2021; Khan *et al.*, 2023). These characteristics highlight the potential of the DT5 as a biocontrol agent against *Colletotrichum* sp. in horticultural systems.

Despite the promising findings, this study has several limitations. Firstly, the DT5 strain has not been identified at the molecular level, which restricts both its precise taxonomic classification and the preliminary evaluation of its biocontrol potential. Secondly, the antifungal role of the DT5 strain against *Colletotrichum* sp. R2 and R3 strains has not been evaluated under field conditions, which limits the assessment of its potential for bioproduct development.

CONCLUSION

Seventeen actinomycete strains were isolated from different collected soil samples. After screening for antifungal ability against the R2 fungi causing anthracnose and the R3 fungus causing leaf spot on rose plants, a potential actinomycete DT5 strain was selected. Based on the morphological characteristics of the colonies, including aerial and substrate mycelia as well as spore chain formation, the actinomycete DT5 strain was preliminarily identified as belonging to the genus *Streptomyces* sp. The DT5 strain showed robust growth on Gause's N°. 1 medium, with a pH range of 5.0-7.0, at temperatures between 30 and 37°C. However, the efficacy of antifungal substances against the R2 and R3 fungi was increased when the strain was cultured in the medium with a pH of 6.0 at 30°C and after 5 days of culture. This strain demonstrated the ability to synthesize cellulase and effectively assimilate glucose and sucrose as carbon sources, along with $NaNO_3$, beef extract, and peptone as nitrogen sources. Our findings provide an important basis for further *in vivo* experiments and biosafety evaluations of the actinomycete DT5 strain. This will help guide the development of effective biological products for agriculture, specifically targeting the management of fungal diseases in roses and potentially extending to other crops.

ACKNOWLEDGMENT

We would like to thank our colleagues of the Biotechnology Faculty, Vietnam National University of Agriculture for their cooperation and kind support throughout our research period.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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