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Antifungal potential of *Streptomyces* sp. CNXK31.2 against strawberry leaf spot pathogen *Mucor* sp. MD7

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ABSTRACT

Control of fungal plant diseases using antagonistic microorganisms is a promising strategy. This study aimed to identify and characterize an antagonistic microorganism effective against *Mucor* sp. MD7, a newly confirmed causative agent of leaf spot disease in strawberry in Da Lat, Vietnam. Koch's postulate was used to confirm *Mucor* sp. MD7 as the causative agent. Antifungal activity screening was conducted through co-culture of 30 actinomycete strains with *Mucor* sp. MD7. The most potent strain was identified using macroscopic and microscopic characteristics, and 16S rRNA sequencing. Enzyme production, antifungal activity under various conditions, and *in situ* tests on strawberry leaves were performed to evaluate the antagonistic potential. Eighteen out of 30 tested actinomycete strains showed antagonistic activity, with Actinomycete CNXK31.2 being the most potent. This strain was identified as *Streptomyces* sp. CNXK31.2, showing 100% identity to *Streptomyces fungicidicus*. It produced chitinase, protease, and cellulase, causing swelling and degradation of *Mucor* sp. MD7 mycelia. The culture supernatant maintained antifungal activity at up to 80°C and pH 3.0–10.0. *In situ* tests demonstrated effective control of leaf spot disease. The findings of this study not only highlight the potential of *Streptomyces* sp. CNXK31.2 as an effective biocontrol agent but also contribute to achieving sustainable development objectives by promoting eco-friendly agricultural practices, safeguarding biodiversity, and enhancing food security.

1. INTRODUCTION

Strawberries, known for their delicate nature, require well-controlled growing conditions. Variations in these conditions and field stresses create favorable environments for various pathogens, including fungi, bacteria, and viruses, which can adversely affect strawberry growth, yield, and fruit quality. In 2021, global strawberry cultivation covered approximately 390 thousand hectares, yielding more than 9 million tons of fruit [1]. Despite its economic importance, strawberry farming faces substantial losses, often reaching several hundred million USD/euro annually, primarily due to damage caused by insects, nematodes, viruses, and bacteria, especially fungi [2]. Leaf spot disease, first reported in October 1952 in Niglar, India, has been a common issue in strawberry crops and significantly impacts production. Pathogens responsible for strawberry leaf spot diseases are increasingly being identified, such as *Exserohilum rostratum* causing strawberry leaf spot disease in China [3], *Corynespora cassiicola* in Taiwan [4], and *Pilidium* sp. in Vietnam [5]. In addition, *Neogestalotiopsis rosae*, a newly discovered pathogen of strawberries, is responsible for leaf blight disease, root rot, and crown rot in strawberries in Taiwan and Central Mexico, leading to losses of up to 50% of transplanted plants [6,7].

To combat these diseases, chemical fungicides, such as carbendazim, mancozeb, copper oxygen chloride, copper hydroxide, propined, fosetyl, thiabendazole, and azoxystrobim, are commonly used, demonstrating rapid and effective results against agents such as *Neoestalotiopsis clavispore*, *C. cassiicola*, *Pestalotia* sp., and others [8–10]. However, fungicides pose challenges in terms of side effects that are challenging to manage, and they have adverse impacts on human health and the environment. Biological control emerges as a sustainable strategy for the strawberry cultivation industry as studies have reported the application of *Trichoderma* spp. to control strawberry leaf spot, leaf blight, and gray mold [9–12]. Bacteria, such as *Bacillus* and *Paenibacillus* [11, 13], along with yeasts [14], have also been recognized as antagonists against various fungal diseases in strawberries. *Streptomyces*, a genus of Gram-positive bacteria known for producing antibiotics, secondary metabolites, and growth-promoting factors, has been investigated for controlling strawberry diseases as well. Elshafie and Camele [15] isolated actinomycete strains that promoted the growth of tomato seedlings and controlled the pathogenic *Sclerotinia sclerotiorum* [15]. In addition, Yong *et al.* [16] reported that *Streptomyces* sp. sdu1201 exhibited significant antifungal activity against *Botrytis cinerea*, the causative agent of strawberry

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gray mold, a severe disease affecting strawberries in both pre- and post-harvest periods [16].

In Vietnam, research on strawberry diseases is limited. Recently, we have isolated several mold strains responsible for leaf spot disease in strawberries in Da Lat and thus expanded our understanding of pathogens affecting this nutrient-rich crop in Vietnam [17]. Among these pathogens, the mold strain *Mucor* sp. MD7 was confirmed as a new causative agent in this study, while most species of this genus are typically associated with postharvest fruit rot. Furthermore, the antagonistic characteristics of *Streptomyces* sp. CNXK31.2 against this pathogenic fungus highlight its potential as a biocontrol agent in strawberry cultivation, aligning with global efforts to achieve sustainable development by reducing reliance on chemical fungicides and promoting biological alternatives that support environmental sustainability and food security.

2. MATERIALS AND METHODS

2.1. Microbial Strains and Culture Condition

The fungal *Mucor* sp. MD7 was isolated and purified from diseased leaf samples collected in Da Lat, Lam Dong province, Vietnam [17]. The fungal strain was cultured at 25°C on potato dextrose agar (PDA) or potato dextrose broth (PDB), while actinomycete strains were cultured at 25°C in Gausse I medium for subsequent experiments.

2.2. Pathogenicity Test on Strawberry Leaves

Healthy strawberry leaves collected from Da Lat, Lam Dong province, Vietnam, underwent a thorough cleaning process, involving three gentle washes with sterile distilled water, surface sterilization with ethanol 70% for 2 minutes followed by five rinses with sterile distilled water, and air dried in a sterile condition. Subsequently, 20 µl of *Mucor* sp. MD7 culture, cultivated for five days PDB, was injected onto the leaf surface. The same procedure was performed for a control group using 20 µl of sterile PDB. The leaves were placed on moistened filter papers in sterilized Petri dishes at 25°C. Disease symptoms were observed daily and compared with those previously recorded [10].

2.3. *In Vitro* Screening of Actinomycete Antagonists

A total of 30 actinomycete strains systematically isolated from soil samples collected across multiple provinces in Vietnam, specifically Binh Thuan, Ninh Thuan, Tay Ninh, Tien Giang, Ben Tre, and Long An, were used in this study. These microbial strains were preserved in the microorganism culture collection maintained by the Institute of Biotechnology and Food Technology at the Industrial University of Ho Chi Minh City, Vietnam. To assess the antagonistic activity against *Mucor* sp. MD7, agar well diffusion method was employed. In brief, the actinomycete strains were cultured in Gause I medium at 37°C for 5 days. Subsequently, 0.1 ml of the culture was inoculated into the wells on PDA plates that had previously been inoculated with MD7. Antagonistic activity was quantified as: antifungal activity (%) = [(colony radius of the control − colony radius of the treatment)/colony radius of the control] × 100%.

2.4. Identification of the Antagonistic Actinomycete

The actinomycete strain with the strongest fungal inhibition was identified and performed through macroscopic and microscopic morphological characteristics, in addition to analysis of 16S rRNA gene region using the universal primer pair 27F-1540R. Homologous gene sequences were obtained from GenBank and a phylogenetic tree was constructed by Mega 11 software, utilizing the neighbor-joining method with 1000 bootstrap repetitions.

2.5. Extracellular Enzyme Assay of the Antagonistic Actinomycete

A 3-µl suspension of 5-day actinomycete culture was inoculated onto a modified Gause I plate (without soluble starch) containing the respective substrates colloidal chitin, casein, and carboxymethyl cellulose (CMC) for chitinase, protease, and cellulase activity. Enzymatic activity unit (AU) was assessed by calculating the ratio of the halo zone diameter to the colony diameter [14].

2.6. Effect of Actinomycete Culture Supernatant on the Fungal Mycelia

The antagonistic actinomycete was cultivated in Gause I broth for 5 days and the culture supernatant was obtained by centrifuging at 13,000 rpm for 10 minutes at 4°C. To prepare the fungal mycelia, the *Mucor* sp. MD7 pathogen was cultured in PDB for 5 days. Subsequently, the actinomycete culture supernatant was added to the *Mucor* sp. MD7 culture in equal volume followed by incubation at 25°C. A mixture in the same ratio of sterile Gausse I broth and *Mucor* sp. MD7 culture was used as a control. The impact on the mycelial morphology was evaluated after 24 hours of incubation under a light microscope.

2.7. Effect of Temperature and pH on Antifungal Activity of Actinomycete Culture Supernatant

To investigate the effect of temperature, 1.0 ml of the culture supernatant was subjected to different conditions 50–100 and 121°C for 30 minutes. To explore the influence of pH, 1.0 ml of the culture supernatant was adjusted to varying pH levels within the range of 3.0–12.0 using different buffers: citric acid/sodium citrate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–7.0), Tris amino methane/hydrochloric acid buffer (pH 8.0–9.0), sodium bicarbonate/sodium hydroxide buffer (pH 10.0), and sodium phosphate dibasic/sodium hydroxide buffer (pH 11.0–12.0). After adjustments, the samples were incubated for 2 hours at room temperature. Subsequently, 20 µl of the treated supernatant was loaded in the wells of PDA plates that had been inoculated with *Mucor* sp. MD7. Similar positive control experiments were conducted, with the negative control using nontreated culture supernatant. Thermal stability and pH stability were evaluated by determining the remaining mycelial growth inhibition percentage in each treatment relative to the control.

2.8. *In Situ* Test of Fungal Antagonistic Activity

Healthy young strawberry leaves, without any mechanical damage or signs of disease, were carefully collected based on their uniform color and size, from a planting farm in Da Lat. A rigorous sterilization process was employed, which involved two rounds of treatment with 70% ethanol, a 2-minute exposure to 2.0% sodium hypochlorite, followed by five times rinsing with sterilized distilled water.

In addition, suspensions of the fungal pathogen and the expected actinomycete were prepared as described in the previous section. Strawberry leaves were placed in sterilized Petri dishes and subjected to four conditions: spraying with 500 µl of sterilized mixture of PDB and Gause I in equal ratio (negative control), spraying with 500 µl of the actinomycete suspension (healthy control), spraying with 500 µl of mixture of the actinomycete suspension and the fungal pathogen suspension in equal ratio, and spraying with 500 µl of the fungal pathogen suspension (infected control). For each condition, five

leaves were used as replicates. The treated leaves were then incubated for 3 days at 25°C ± 2°C with disease progression being monitored daily [10].

2.9. Data Analysis

All the experiment was done in triplicate except indicated elsewhere. The data were visualized using Microsoft Excel and ANOVA tests (α = 0.05) were done using Statgraphics Centurion 18 software (Statgraphics Technologies, Inc., USA).

3. RESULTS AND DISCUSSION

3.1. Confirmation of the Strawberry Leaf Spot Pathogen *Mucor* sp. MD7

The fungal strain *Mucor* sp. MD7 was isolated from infected strawberry leaves originating from farms in Da Lat, Lam Dong province, Vietnam. Macroscopic and microscopic characteristics are depicted in Figure 1A. Following Koch's postulates method, it was confirmed that *Mucor* sp. MD7 causes disease to healthy strawberry leaves after 6 days of infection with typical leaf spot symptoms. The leaf tissues of both sides became deep brown, exhibited necrosis, and developed disease manifestations rapidly during a 6-day incubation period at 25°C ± 2°C (Fig. 1C). In contrast, the control sample, injected with sterilized PDB, showed no disease symptoms (Fig. 1B).

The disease induced by *Mucor* sp. MD7 exhibits similarities with previously reported cases of other strawberry leaf spot fungal pathogens such as *G. fragariae* [18], *E. rostratum* [3], and *C. cassiicola* [4], suggesting a diversity of strawberry leaf pathogens. In addition to documented instances of *Mucor* sp. causing fruit rot, this marks the initial report of a *Mucor* sp. strain responsible for leaf spots in strawberries in Vietnam.

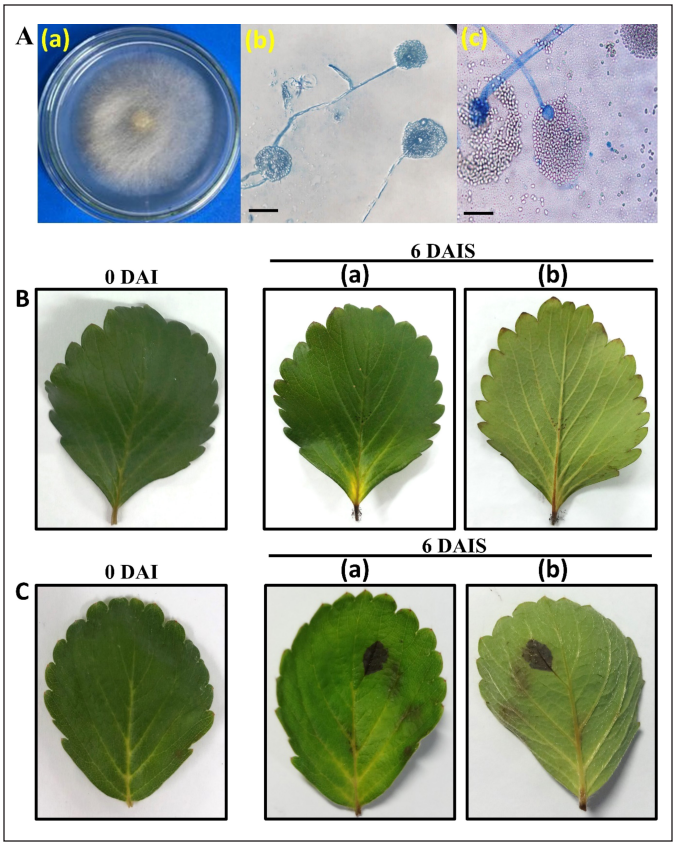

Figure 1. Strawberry leaf spot caused by *Mucor* sp. MD7. (A) Fungal pathogen with macromorphology (a) and micromorphology (b, c). (B) Healthy and (C) infected leaf with upper side (a) and lower side (b). DAI: day after infection. Scale bar = 50 µm.

3.2. *In Vitro* Screening of Antifungal Actinomycetes

The findings revealed that 18 out of 30 actinomycetes strains exhibited antifungal activity against *Mucor* sp. MD7, causing varied mycelial inhibition ranging from 4.55% to 36.4% (Fig. 2A). Notably, the strain CNXK31.2 demonstrated significant antifungal activity with the highest inhibition rate of 36.4% (Fig. 2B). This achieved fungal inhibition percentage surpasses the inhibitory rates of 24 yeast strains against *B. cinerea* on strawberry fruit, as determined by the agar diffusion method [14]. In addition, it is on par with the inhibitory effect on *C. sojina* growth observed in *Streptomyces* sp. MM140, as determined by the dual assay method [19]. Given robust and consistent mycelial inhibition activity against *Mucor* sp. MD7, CNXK31.2 was chosen for further investigation.

3.3. Identification of Antagonistic Actinomycete CNXK 31.2

The identification of actinomycete strain CNXK31.2 involved an examination of its microscopic, macroscopic, and 16S rRNA characteristics. After 5 days of cultivation on Gause I medium at 37°C, colonies were round with a diameter of 3–4 mm, brownish-gray color, regular edges, raised centers, and numerous small gaps on the surface along with reddish-brown pigment production (Fig. 3A-a). Microscopic observation revealed branching mycelia and spore-bearing aerial hyphae. The retinaculiaperti spore chain was spiraled, short, and cleaved into conidia as it matured (Fig. 3A-b).

The 16S rRNA sequence, consisting of 1,306 bp, was amplified and sequenced. The BLAST results revealed that CNXK31.2 exhibited a high sequence identity with *S. fungicidicus* NBRC 13848 and *S. fungicidicus* A1 (100%). Furthermore, the phylogenetic tree analysis

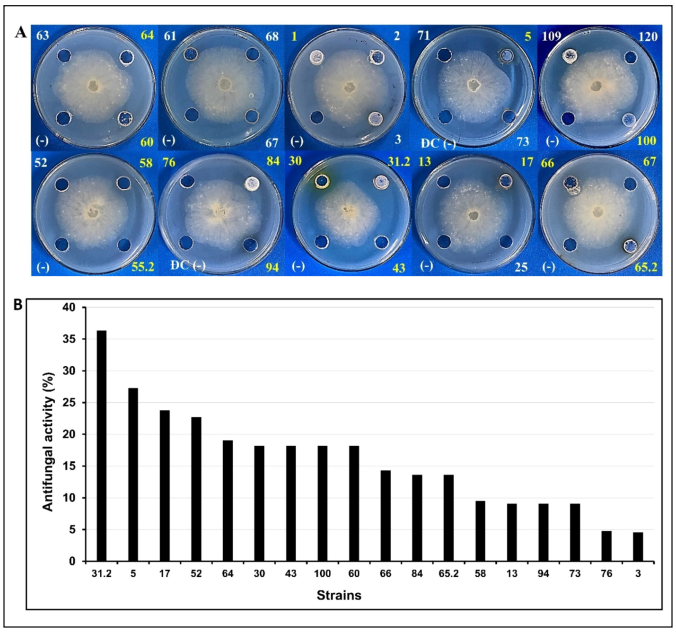

Figure 2. *Mucor* sp. MD7 mycelial inhibition of actinomycetes. Different numbers represent different strains, (−) negative control.

positioned CNXK31.2 in the genus *Streptomyces* and in the same clade as *S. fungicidicus* NBRC 13848 with 71% similarity (Fig. 3B). *Streptomyces fungicidicus* strains have been previously reported to produce an antibiotic, enduracidin, which acts against methicillin-resistant *Staphylococcus aureus* [20]. It is worth noting that *S. fungicidicus* was also found to exhibit biocontrol effects on pathogenic fungi such as *Alternaria solani* and *Sclerotium rolfsii* [21,22]. Thus, actinomycete strain CNXK31.2 was named *Streptomyces* sp. CNXK31.2 and 16S rRNA sequence data were deposited at NCBI GenBank with accession numbers PQ146974.1.

3.4. Extracellular Enzyme Activity of *Streptomyces* sp. CNXK31.2

The results presented in Figure 4 and Table 1 demonstrated that CNXK31.2 exhibited the production of extracellular hydrolytic enzymes, including 1.4 ± 0.2 AU of chitinase activity, 3.2 ± 0.3 AU of protease activity, and 1.7 ± 0.2 AU of CMCase. The biosynthesis of chitinase, protease, and cellulase, known for their role in breaking down fungal cell walls, has been observed in various fungal antagonists, such as actinomycete strains isolated from the rhizosphere of strawberry, rosemary, acacia, and olive plants for controlling *S. sclerotiorum* on tomato plants [15], *Bacillus velezensis* CE 100 combating anthracnose caused by *Colletotrichum gloeosporioides* [23], and *Bacillus safensis* B3 for controlling *B. cinerea* gray mold in strawberry fruits [24]. In addition, yeast antagonists have also shown similar hydrolytic enzyme production when combating *B. cinerea* on strawberry fruits [14]. Thus, these results suggest that *Streptomyces* sp. CNXK31.2 possesses a robust capacity for hydrolytic enzyme production, potentially contributing to its ability to inhibit fungal pathogens. This offers an eco-friendly alternative to chemical fungicides, avoiding environmental toxicity and resistance development associated with chemical use.

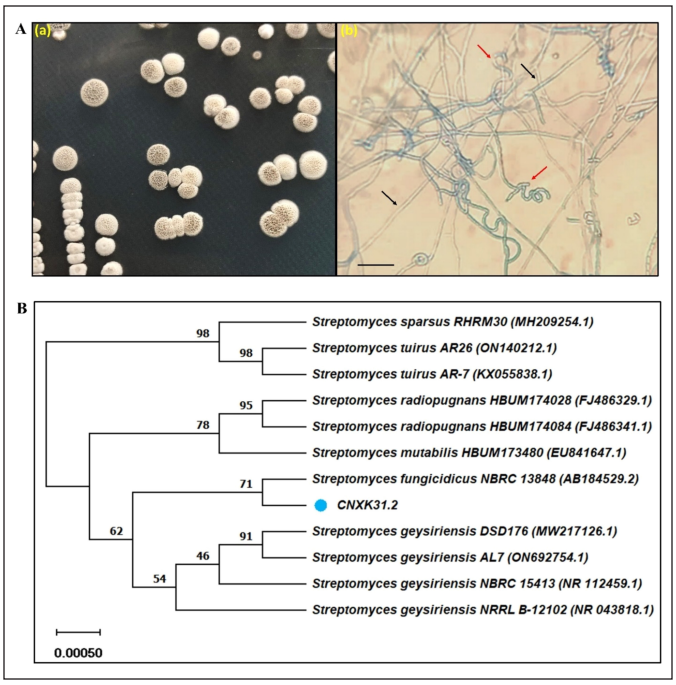

Figure 3. Identification of actinomycete CNXK31.2. (A-a) Colony morphology and (A-b) vegetative hyphae (black arrows), spore-bearing aerial hyphae (white arrows). (B) Phylogenetic tree of CNXK31.2 based on 16S rRNA sequence. Numbers on branch nodes are bootstrap values (expressed in percentage). Scale bar = 10 µm.

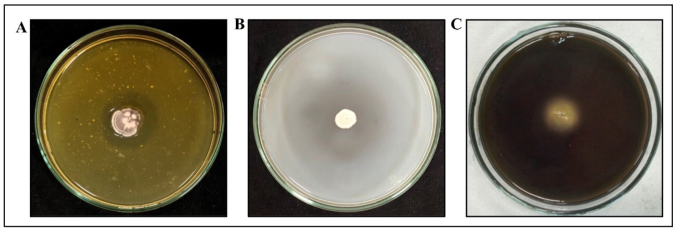

Figure 4. Hydrolytic activity of CNXK31.2. (A) Chitinase. (B) Caseinase. (C) CMCase.

Table 1. Hydrolytic enzyme activity of CNXK31.2.

	Diameter of the halo zone (mm)	Diameter of the colony (mm)	Enzyme activity* (AU)
Chitinase	5.0 ± 0.4	3.5 ± 0.5	1.4 ± 0.2
Protease	9.7 ± 0.8	3.0 ± 0.5	3.2 ± 0.3
CMCase	4.1 ± 0.4	2.5 ± 0.5	1.7 ± 0.2

*Enzyme activity unit (AU) is calculated as diameter of the halo zone/diameter of the colony.

3.5. Effect of *Streptomyces* sp. CNXK31.2 Culture Supernatant on the Fungal Mycelia

After a 24-hour inoculation, in the control group without the *Streptomyces* sp. CNXK31.2 culture supernatant, *Mucor* sp. MD7 mycelia exhibited normal growth with long, thick, and branched mycelia (Fig. 5A). On the contrary, in the presence of culture supernatant, the fungal mycelia displayed aberrant morphology, including deformed, thinning, or swollen of hyphae, and even hyphae degradation (Fig. 5B). Irregularity observed in the pathogenic MD7 mycelia when treated with the CNXK31.2 culture supernatant can be attributed to cell wall degradation facilitated by hydrolytic enzymes in the culture. These findings are in line with the study of Lee *et al.* [25], where the culture solution of *Streptomyces cavourensis* SY224 had a similar influence on the mycelium of *C. gloeosporioides* after a 72-hour inoculation. In addition, compounds produced by *Streptoverticillium morookaense* demonstrated comparable effects on the mycelial morphology of phytopathogenic fungi, including *Ustilaginoidea virens*, *Rhizoctonia solani*, and *Bipolaris maydis* [26].

3.6. Effect of Temperature and pH on Antifungal Activity of *Streptomyces* sp. CNXK31.2

The culture supernatant of *Streptomyces* sp. CNXK31.2 demonstrated thermal stability concerning its antifungal activity within the temperature range of 50°C–80°C, with a remaining antagonistic activity of 75.0%–95.8% compared to the untreated control (Fig. 6A). Exposure to temperatures exceeding 90°C resulted in the complete loss of antifungal activity, suggesting a marginally less stable than in the case of *Streptomyces albulus* Z1-04-02 culture supernatant with ~34% remaining activity at 100°C [27]. In addition, the culture supernatant displayed robust stability in neutral and alkaline environments, with antifungal activity exceeding 80% at pH levels between 7.0 and 10.0. At acidic pH levels of 3.0–6.0, the activity considerably remained at 39.6%–66.7% (Fig. 6B). Similar observation in antifungal activity of *Streptomyces* sp. TQR12-4 culture supernatant against *Geotrichum candidum*, *F. oxysporum*, and *F. udum* [28]. Consequently, *Streptomyces* sp. CNXK31.2 demonstrated both broad-spectrum pH stability (pH 3.0–10.0) and thermal stability (50°C–80°C), making it a potential candidate for the development of a safe bio-fungicide.

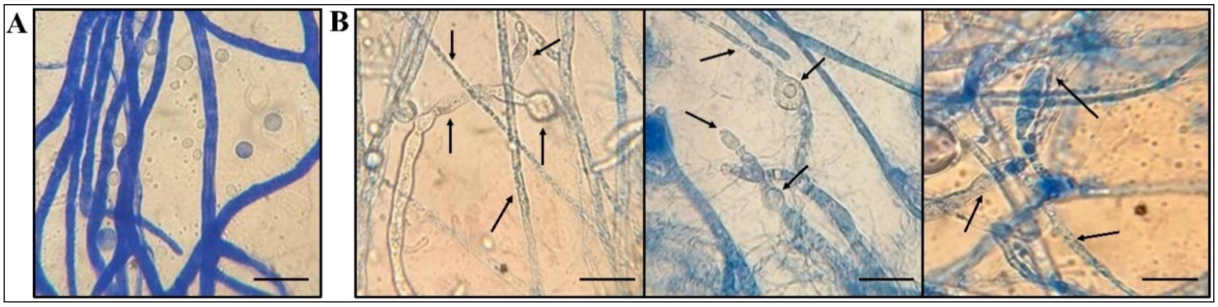

Figure 5. Effect of CNXK31.2 culture supernatant on MD7 mycelia. (A) Control and (B) treatment. Black arrows indicate sites of mycelial abnormality. Scale bar = 10 µm.

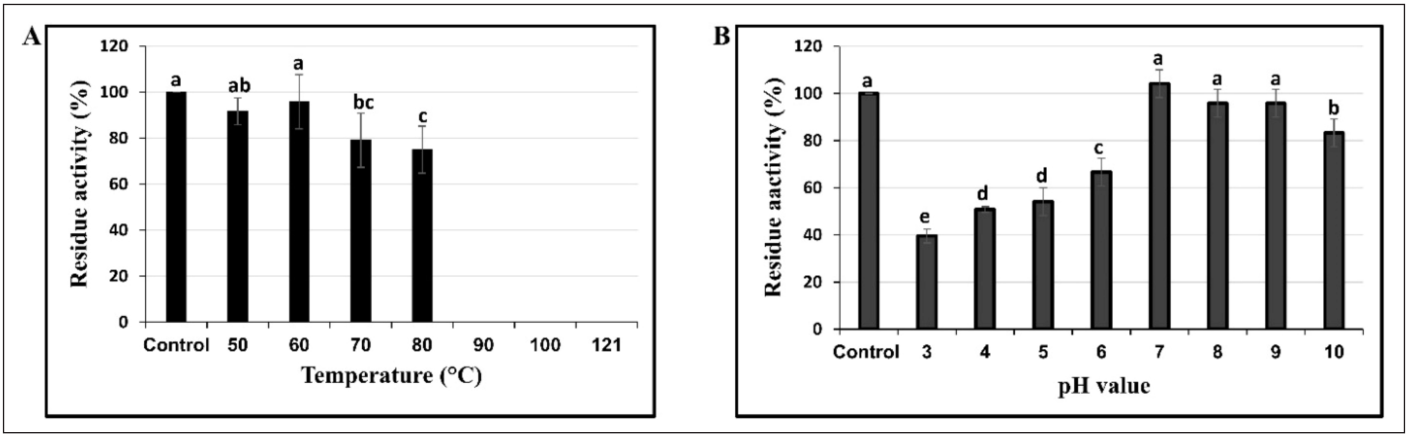

Figure 6. Effect of temperature (A) and pH (B) on stability of antifungal agents against *Mucor* sp. MD7. Means with different letters above columns statistically differ from each other ($p < 0.05$).

3.7. *In Situ* Test of Fungal Antagonistic Activity on Strawberry Leaf Model

After 1 day of inoculation, disease symptoms, characterized by black spots on strawberry leaves injected with *Mucor* sp. MD7, were visible compared to no observable symptom on leaves of other conditions (Fig. 7A). After 3 days of inoculation, the black spots began to spread, and the symptoms gradually extended as the leaf tissues exhibited necrosis as the mold prominently proliferated. In contrast, strawberry leaves treated with a mixture of CNXK31.2 and MD7 displayed no differences in leaf surface compared to the control with sterilized media. Furthermore, leaves treated with *Streptomyces* sp. CNXK31.2 exhibited no signs of disease symptoms but mycelial proliferation (Fig. 7B). This suggests that *Streptomyces* sp. CNXK31.2 is not pathogenic to strawberries and is capable of inhibiting leaf spot caused by *Mucor* sp. MD7.

The utilization of microbial strains as biocontrol agents for the protection and management of strawberry leaf diseases has been getting more attention. For instance, the application of *Trichoderma asperellum* has been found to inhibit mycelial growth and reduce the appearance of leaf blight caused by *Neopestalotiopsis clavispora* in strawberries [9]. Similarly, reports indicate the effectiveness of *Aureobasidium pullulans* in decreasing the disease severity index associated with *Mycosphaerella fragariae*, a leaf spot pathogen [29]. Furthermore, *Streptomyces* sp. 3–10 crude extract has demonstrated the suppression of various fungal pathogens affecting strawberries, including *B. cinerea*, *Mucor hiemails*, *Saxifraga stolonifera*, and *S. sclerotiorum* [30]. These findings underscore the potential application of the actinomycete strains, including the one under study in the prevention of strawberry diseases.

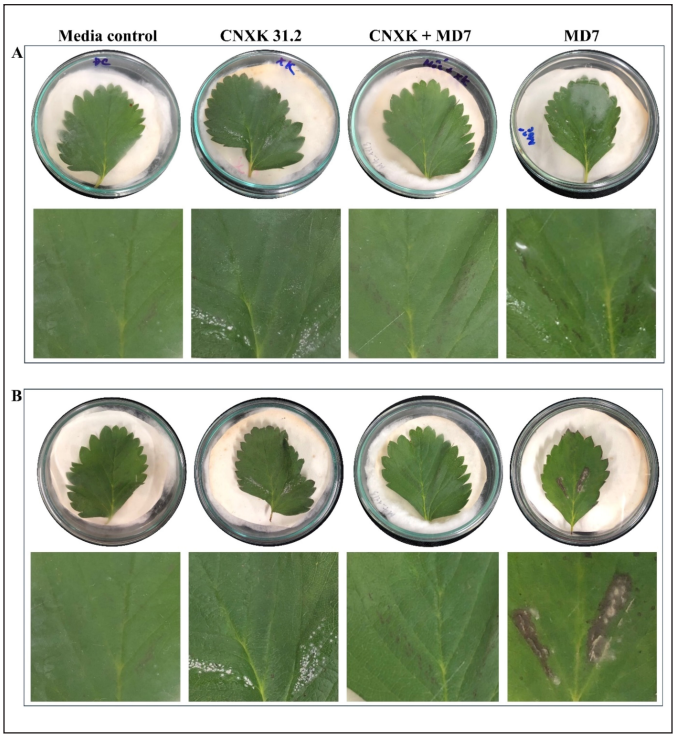

Figure 7. *In situ* test of *Mucor* sp. MD7 antagonistic activity of *Streptomyces* sp. CNXK31.2 on strawberry leaves. (A) After 1 day and (B) after 3 days of infection.

4. CONCLUSION

In summary, this is the first report on *Mucor* sp. causing leaf spot in strawberry in Vietnam and the application of actinomycetes strains to control this fungal pathogen. As effective pathogen control is a pivotal aspect of modern cultivation practices, *Streptomyces* sp. CNXK31.2 showed remarkable prowess in impairment of the pathogenic mycelial growth and structural damage of *Mucor* sp. MD7. Notably, *Streptomyces* sp. CNXK31.2's culture supernatant displayed high thermal stability and broad-spectrum pH stability, underscoring the potential use of this strain in diverse environmental conditions. In the strawberry leaf model, *Streptomyces* sp. CNXK31.2 effectively controlled leaf spot disease caused by *Mucor* sp. MD7 while alone, causing no harm to the leaf. Studies on metabolomic profiling, detailed enzyme mechanisms, interaction with host plants, and long-term impacts on soil microbiota are necessary to fully understand the antifungal mechanism. Consequently, the findings of this study not only highlight the potential of *Streptomyces* sp. CNXK31.2 as an effective biocontrol agent but also contribute to achieving sustainable development objectives by reducing chemical fungicide pollution and promoting long-term soil health.

5. LIST OF ABBREVIATIONS

AU, activity unit; CMC, carboxymethyl cellulose; PDA, potato dextrose agar; PDB, potato dextrose broth.

6. ACKNOWLEDGMENT

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. FUNDING

There is no funding to report.

9. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the data are available with the authors and shall be provided upon request.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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