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Characterisation of antagonistic actinomycetes against *Neoscytalidium dimidiatum* causing stem canker on dragon fruit (*Hylocereus* spp.)

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ABSTRACT

Dragon fruit (*Hylocereus* spp.) is a high-value crop in tropical and subtropical agriculture; however, its productivity is severely constrained by stem canker and fruit rot induced by *Neoscytalidium dimidiatum*. The present study was conducted to investigate the sustainable biocontrol strategies to mitigate phytopathological challenge caused by *N. dimidiatum* on dragon fruit. Fifty (n=50) actinomycete isolates were evaluated out of which 36 exhibited antagonistic effects against *N. dimidiatum*. Among them, strain CNXK121 exhibited the highest antifungal potency. Based on morphological characterisation and 16S rRNA gene sequencing, strain CNXK121 was identified as *Streptomyces plicatus* (100% sequence identity) and designated as *Streptomyces* spp. CNXK121. The antagonistic activity of CNXK121 was attributed to its production of cell-wall-degrading enzymes (CWDEs), specifically chitinase and protease, alongside the emission of inhibitory volatile organic compounds (VOCs). Microscopic observations revealed that CNXK121 treatments effectively suppressed conidial germination and induced marked mycelial distortions in *N. dimidiatum*. Furthermore, *in vivo* assays on dragon fruit stems and fruits further confirmed the efficacy of *Streptomyces* spp. CNXK121 in reducing disease severity. These results highlight the potential of *Streptomyces* spp. CNXK121 as a robust biological control agent (BCA) for integrated disease management and the sustainable cultivation of *Hylocereus* spp. in Vietnam.

Keywords: Antifungal, Dragon fruit, *Neoscytalidium dimidiatum*, Pitaya, *Streptomyces*

Neoscytalidium dimidiatum is a highly adaptable fungal pathogen with an extensive host range across various plant families. It is notorious for causing a wide array of diseases, including fruit and root rot, leaf spot, blight, stem canker and dieback, infecting more than 126 host plant species (Derviş and Ozer 2023). One of the most economically significant hosts of *N. dimidiatum* is dragon fruit (*Hylocereus* spp.) also known as pitaya. This pathogen causes stem and fruit canker in dragon fruit, reported across Asia and America (Serrato-Diaz and Goenaga 2021, Salunkhe *et al.* 2023, Espinoza-Lozano *et al.* 2023). This disease reduces plant vigour and fruit quality, often leading to severe yield losses and posing a major threat to dragon fruit growers.

Fungicides like azoxystrobin, difenoconazole, mancozeb and copper are widely used against *N. dimidiatum* in dragon fruit but pose environmental and health risks (Noegrohati *et al.* 2019), highlighting the need for alternatives. Biological control offers a sustainable strategy for managing dragon fruit cultivation. Wang *et al.* (2021) showed that the endophytic fungus *Penicillium rolfsii* Y17 effectively controlled pitaya canker with a 70.87% inhibition rate, while Lin *et al.* (2023) reported that *Paenibacillus polymyxa* AF0 reduced disease indices up to 50% at 14 days after post-inoculation compared to controls.

Actinomycetes are gram-positive bacteria known for producing a wide range of antibiotics, have shown potential in controlling several fungal pathogens including *N. dimidiatum* (Al Hamad *et al.* 2021). However, research on the application of actinomyces manage diseases caused by *N. dimidiatum* on pitaya remains scare. In this study, the antifungal activity of *Streptomyces* spp. CNXK121, selected from 50 actinomycetes strains, was evaluated against *N. dimidiatum*.

MATERIALS AND METHODS

Microbial strains and culture conditions: The present study was carried out during 2024–25 at Institute of Biotechnology and Food technology, Industrial University of Ho Chi Minh City, Vietnam. The fungus strain, *Neoscytalidium dimidiatum* and fifty (n=50) actinomycete strains were obtained from the Microbiotechnology Laboratory, Institute of Biotechnology and Food technology, Industrial University of Ho Chi Minh City, Vietnam. The

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fungal strain was cultured on PGA (potato glucose agar) medium at 30°C, while the actinomycete strains were cultured in the Gause I medium at 37°C, shaken at 150 rpm for five-days and stored at 4°C for further experiments.

In vitro *screening of fungal antagonistic actinomycetes*: The antagonistic activity of 50 actinomycete strains against the pathogenic fungus *N. dimidiatum* was examined by agar well diffusion method (Ansari *et al.* 2021). Actinomycete strains were cultured after which the cultures were harvested and centrifuged at 10,000 rpm at 4°C for 5 min to obtain the supernatants. Fifty microliters of culture supernatant were introduced into wells on PGA plates that had been pre-inoculated with *N. dimidiatum* one day prior. A control well was prepared using sterilised Gause I medium. Inhibition zone (I) was measured after four days of incubation using the given formula ((Zhang *et al.* 2020):

Inhibition zone (I) (mm) = Longest fungal radius - Inhibited fungal radius

The actinomycete strain exhibiting the strongest antifungal activity was selected for all subsequent biocharacterisation experiments.

Identification of the antagonistic actinomycete: The actinomycete strain showed the highest fungal inhibition was identified based on macro-microscopic morphological characteristics, along with 16S rRNA gene sequence analysis. The 16S rRNA gene region was amplified and sequenced with universal primer pair 27F: 5'-AGAGTTTGATCMTGCTCAG-3' and 1540R: 5'-AAGGAGGTGATCCAACCGCA-3'. Homologous gene sequences were retrieved from Genbank (National Centre for Biotechnology Institute) and aligned using Clustal X2.1. A phylogenetic tree was constructed based on the neighbour joining algorithm with 1000 bootstrap repetitions using MEGA11 software (Fredriksson *et al.* 2013)**.**

Hydrolytic enzyme assays of the antagonistic actinomycete: Enzyme hydrolytic activities were assessed by cultivating the selected actinomycete strain on solid media containing the corresponding substrates. Chitinase and protease were evaluated using Gause I medium, in which the starch component was replaced with colloidal chitin and casein, respectively. A 3 µL actinomycete culture was inoculated onto the prepared plates and incubated at room temperature for seven days. Protease activity was visualised using a 10% trichloroacetic acid solution, while chitinase and cellulase activities were assessed using Lugol's solution. Enzyme activity (AU) was measured using the given formula (Chen *et al.* 2018):

Enzyme activity (AU) (mm) = Halo zone diameter - Colony diameter

Effect of actinomycete VOCs and cell culture on fungal development: The antagonistic activity of volatile organic compounds (VOCs) produced by the selected actinomycete strain against *N. dimidiatum* was assessed using a two-compartment Petri dish. The fungal pathogen and the antagonistic actinomycete were cultured separately, preventing physical contact while allowing VOCs to diffuse. The fungal pathogen was inoculated on PGA medium in one section of the dish, while the antagonistic actinomycete was cultured on Gause I medium in the other section. The plate was sealed with parafilm to maintain a closed environment for VOC diffusion and incubated at room temperature. Control plates were prepared under identical conditions but without the antagonistic actinomycete (Li *et al.* 2020). To assess the effect of cell culture components on fungal development, cultures were centrifuged at 13,000 rpm at 4°C. The cell pellet was washed three times with 0.9% NaCl and then resuspended in 0.9% NaCl. The supernatant was further filtered through a 0.22 µm membrane to obtain a cell-free supernatant. Antifungal activity was evaluated using the agar well diffusion method by adding into each well one of the following, cell-free supernatant, cell suspension, cell culture, Gause I medium, NaCl 0.9% and distilled water. Inhibition zone (I) were recorded after four days of incubation (Li *et al.* 2016).

Effect of actinomycete culture on fungal spore germination and mycelia growth: Fungal spores were prepared by culturing *N. dimidiatum* in PGA tube at 30°C for seven days. Sterilised 0.9% NaCl was then added to the tube, the spore suspension was gently collected and adjusted to a concentration of 10^6 spores/mL. To assess the effect of the actinomycete strain on fungal spore germination, the actinomycete culture and fungal spore suspension were mixed in a 1:1 (v/v) ratio and incubated for 4, 8, 12 and 24 h. At each time point, 0.1 mL of the mixture was spread onto PGA plates to evaluate the fungal growth (Nguyen *et al.* 2020). The effect of the actinomycete culture on fungal mycelia was examined by culturing the fungal pathogen in potato glucose broth (PGB) for five days, followed by the addition of an equal volume of actinomycete culture. The mixture was incubated at 30°C for 24 h and mycelial morphology was observed under a light microscope.

In vivo *test of fungal antagonistic activity of actinomycete culture on pitaya*: The biocontrol potential of the actinomycete against the *N. dimidiatum* was evaluated on pitaya (*H. costaricensis*) fruits and stems. Healthy young pitaya fruits of uniform colour and size, free from mechanical damage and disease were collected from Hong May Farm, Group 3, Dan Thuan Hamlet, Ham Thanh Commune, Lam Dong Province. The fruits were surface sterilised sequentially with 70% ethanol (twice), 2% sodium hypochlorite for 2 min and rinsed five times with sterilised distilled water, followed by air-drying under sterile conditions. Actinomycete suspensions and *N. dimidiatum* spore suspensions were prepared as described above. Sterilised pitaya fruits were placed in sterile Petri dishes and subjected to the following treatments: Inoculated with 200 µL of sterilised media (PDB and Gause I at a 1:1 ratio) as a negative control; Inoculated with 200 µL of actinomycete suspension as a healthy control; Inoculated with 200 µL of fungal spore suspension as an infected control; Inoculated with 200 µL of a mixture of actinomycete suspension and fungal pathogen spores at various ratios (1:0.5, 1:1 and 1:2).

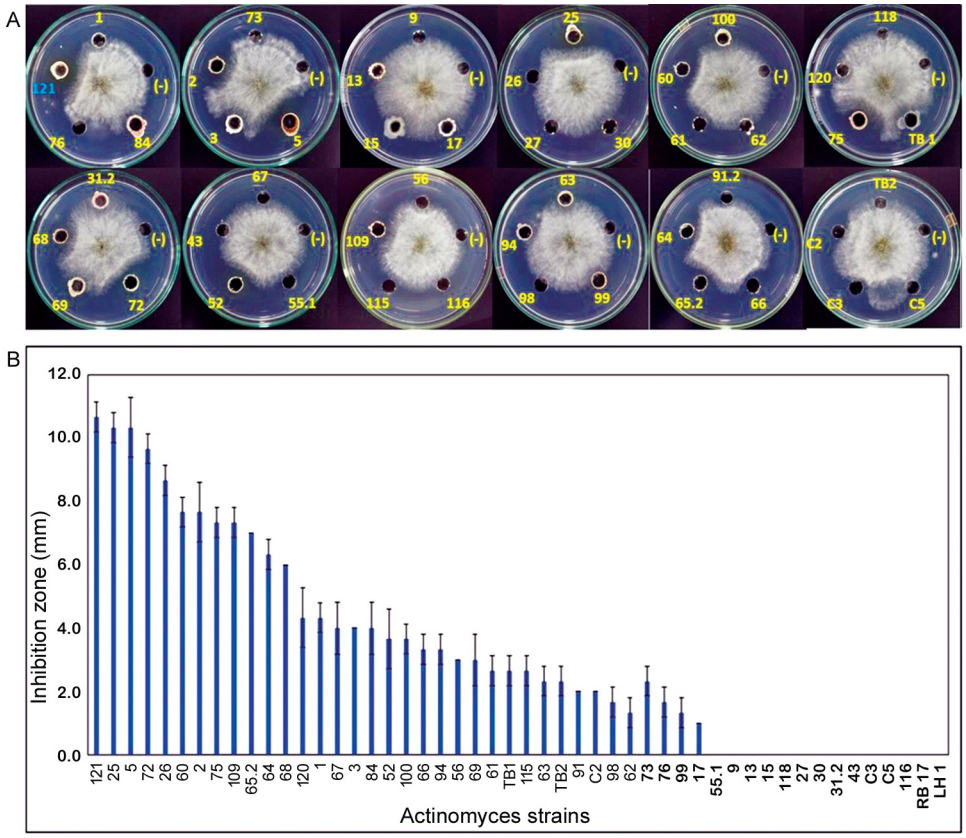

Fig. 1 *N. dimidiatum* growth inhibition by actinomycetes.

Three fruits were used as replicates for each treatment. The treated pitaya fruits were incubated in sterilised Petri dishes at 30°C for five days and disease symptoms were observed daily (Orellana and Mattos 2019). The same experiment was performed on pitaya stems to further evaluate the *in vivo* antagonistic activity of the actinomycete culture (Orellana and Mattos 2019).

Data analysis: All experiments were conducted in triplicate and data visualisation was performed using Microsoft Excel and statistical analysis was carried out using ANOVA tests ($\alpha = 0.05$) in Statgraphics Centurion software (Version 18, Statgraphics Technologies, Inc., USA).

RESULTS AND DISCUSSION

In vitro *screening of antifungal actinomycetes against* N. dimidiatum: Among 50 actinomycete isolates, 36 isolates exhibited antifungal activity with inhibition zones ranging from 1.3±0.6 mm to 10.7±0.5 mm (Fig. 1A). Actinomycete strain CNXK121 was recorded for the highest antifungal activity with an inhibition zone of 10.7±0.5 mm (Fig. 1B). The inhibition zone greater than 10.0 mm is classified as strong antifungal activity (Paul *et al.* 2007). Therefore, CNXK121 was identified as the most potent strain and selected for further identification and investigation of its antifungal properties.

Identification of antagonistic actinomycete CNXK121: Actinomycete strain CNXK121 exhibiting strong inhibition activity against *N. dimidiatum*, was identified based on microscopic, macroscopic and 16S rRNA gene sequence analyses. Morphological observations revealed that after five days of culture on Gause I medium at 37°C, colonies were round, measuring 3–4 mm in diameter with a light-grey colour, regular edge and a depressed centre with no pigmentation and displayed white filaments at the periphery (Fig. 2A-a). At 1000× magnification, branching mycelia and spore-bearing aerial hyphae were observed. The spore chains were spiral-shaped, short and cleaved into individual spores upon maturation (Fig. 2A-b).

The 16S rRNA gene of strain CNXK121 was amplified, sequenced and aligned with nucleotide sequences from bacterial species available in the GenBank. BLAST analysis revealed that strain CNXK121 exhibited 100% sequence identity with *Streptomyces plicatus* NBRC 13071 and clustered in the same clade as *S. plicatus* NBRC 13071 in phylogenetic tree analysis (Fig. 2B). Based on these results, our strain CNXK121 was identified and designated as *Streptomyces* spp. CNXK121. Likewise, previous studies have also reported that different strains of *S. plicatus* possess biocontrol activity against various fungal pathogens, including *Verticillium dahliae*, *Rhizoctonia*, *Sclerotinia*, *Fusarium*, *Rosellinia*, *Pythium* and *Phytophthora* (Abd-Allah 2001, Aghighi *et al.* 2004, Baharlouei *et al.* 2010, Chen *et al.* 2016).

Hydrolytic enzyme activity of Streptomyces *spp. CNXK121*: The fungal cell wall is primarily composed of a complex of chitin, glucan and mannoproteins, whereas the cell wall of oomycetes primarily contains cellulose and glucan (Mélida *et al.* 2013). The production of extracellular hydrolytic enzymes that degrade the fungal cell wall is a key antifungal mechanism of biocontrol microbes. Therefore, the ability of strain CNXK121 to produce extracellular chitinase and protease was assessed.

The results in (Table 1) indicate that *Streptomyces*

Table 1 Enzymatic activities of chitinase and protease of *Streptomyces* spp. CNXK121

	Halo zone diameter (mm)	Colony diameter (mm)	Enzymatic activity (AU)
Chitinase	28.3±1.5	9.3±1.2	30.8±5.8
Protease	13.7±0.6	8.3±0.6	16.4±1.0

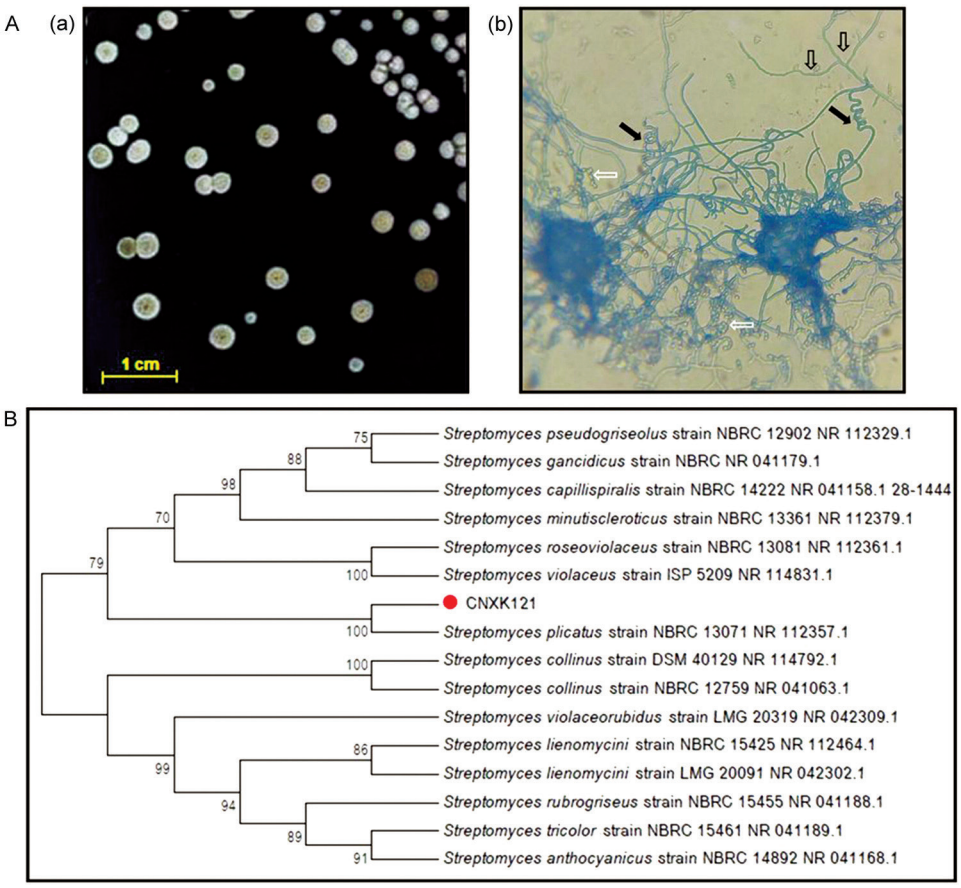

Fig. 2 Characterisation of actinomycete CNXK121. (A) Morphology of colony (a) and mycelia (b) with hyphae (unfilled black arrows), spore-bearing aerial hyphae (black arrows), spore-chain (unfilled white arrows). (B) Constructed phylogenetic tree using 16S rRNA sequences.

spp. CNXK121 exhibits extracellular hydrolytic enzyme biosynthesis, with chitinase activity measured at 30.8 AU, protease activity at 16.4 AU. Indeed, the strong chitinase producing capability of the antifungal antagonist *S. plicatus* has also been observed in previous studies (Abd-Allah 2001, Baharlouei *et al.* 2010). In a study by Wang *et al.* (2015), chitinase from *Streptomyces scabrisporus* Bn03 effectively inhibited *Bipolaris sorokiniana*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium capsica*, which caused root rot in turfgrass. Additionally, the biosynthesis of chitinase and protease has been observed in other fungal antagonists such as *Bacillus velezensis* CE100 (Choub *et al.* 2021), *Bacillus safensis* B3 (Hassan *et al.* 2021) and yeast strains (Chen *et al.* 2018). Therefore, the results suggested that *Streptomyces* spp. CNXK121 had a strong ability to produce chitinase, which may play a key role in its antifungal activity against plant pathogens.

Effect of actinomycetes VOCs and cell culture on fungal development: The ability of actinomycete strain CNXK121 to produce antifungal volatile organic compounds was assessed through co-cultivation in partitioned Petri plates and statistical differences were observed in *N. dimidiatum* mycelial growth as compared to control (Fig. 3). On control plates, *N. dimidiatum* grew densely, secreting a black pigment that covered the entire medium surface (Fig. 3A-a) whereas in strain CNXK121, mycelial growth was sparse, with thinner mycelium and black pigment secretion limited to the inoculation site (Fig. 3A-b). These results indicated that VOCs produced by CNXK121 can inhibit *N. dimidiatum* mycelial growth. VOCs typically consist of a complex mixture of low-molecular-weight compounds that readily volatilise under

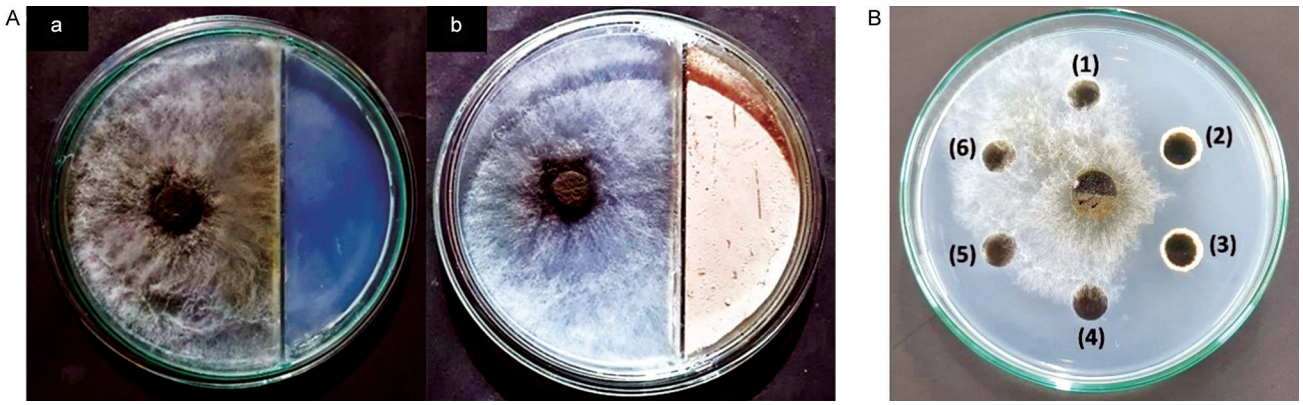

Fig. 3 Inhibition of *N. dimidiatum* mycelial development by VOCs from CNXK121 (A) and CNXK121 culture (B). (a) Control without CNXK121, (b) Co-cultivation of *N. dimidiatum* (left) and CNXK121 (right), (1) CNXK121 cell-free supernatant, (2) Washed CNXK121 cells, (3) CNXK121 culture, (4) Gauze I broth, (5) 0.9% NaCl, (6) Distilled water.

environmental conditions. Certain microorganisms, such as *Streptomyces* and *Bacillus* strains, have demonstrated significant biological activity against plant pathogens through VOC emissions (Cordovez *et al.* 2015, Gotor-Vila *et al.* 2017). Herrington (1987) reported that methyl vinyl ketone (butenone) produced by *Streptomyces griseoruber* inhibits spore germination of *Cladosporium cladosporioides*. Similarly, Wang *et al.* (2013) identified dimethyl disulfide from *Streptomyces alboflavus* TD-1 as a key compound responsible for inhibiting *Fusarium moniliforme* mycelial growth. Later, Wu *et al.* (2015) identified anisole in the VOCs of *Streptomyces albulus* NJZJSA2, which was shown to inhibit *Sclerotinia sclerotiorum* and *Fusarium oxysporum*.

Additionally, the presence of actinomycete cells in the broth is crucial for antifungal activity against *N. dimidiatum*, as demonstrated by the washed cell suspension and cell culture, whereas the cell-free culture supernatant, Gause I broth, 0.9% NaCl solution and sterile distilled water showed no inhibitory effect (Fig. 3B). These findings aligned with those of Wang *et al.* (2020), in which it was reported that the antagonistic mechanism of *Metschnikowia citriensis* against *Geotrichum citri-aurantii* is the causative agent of sour rot in post-harvest citrus fruits. Their study revealed that both washed and unwashed yeast cells significantly inhibited fungal growth, whereas the cell-free culture broth, sterile culture medium and sterile distilled water had no inhibitory effect.

Effect of actinomycete culture on fungal spore germination and mycelia growth: The inhibitory effect of CNXK121 culture on spore germination and mycelial growth varied depending on the incubation duration (Fig. 4A). Following an 8 h incubation period, significant inhibition of fungal biomass was observed, with the concurrent establishment of CNXK121 biomass on the medium surface. The inhibitory effect on spore germination became increasingly pronounced with extended incubation duration. After 24 h of incubation, actinomycete colonies demonstrated vast proliferation across the medium surface, while fungal mycelium was present in negligible quantities.

The effect of *Streptomyces* spp. CNXK121 on *N. dimidiatum* mycelia was further investigated by co-incubating the CNXK121 culture with fungal mycelia and examining morphological changes after 24 h. In the control group, fungal mycelia exhibited normal growth with homogenously long branched structures (Fig. 4B). In contrast, fungal mycelia incubated with CNXK121 displayed irregular morphology, including bending, swelling or thinning and even degradation of the hyphae, which can partially be attributed to cell wall damages induced by chitinase and protease present in the culture (Fig. 4B).

Similar effects have also been reported in previous study, such as *Streptomyces cavourensis* SY224, which caused severe mycelial damage in *Colletotrichum gloeosporioides* (Lee *et al.* 2012). Additionally, antifungal compounds produced by *Streptomyces* strains have been shown to affect fungal mycelia, including antifungalmycin N2 from *Streptomyces* spp. strain N2, which disrupts *Rhizoctonia solani* cell membranes (Zhang *et al.* 2020) or volatile compounds from *Streptomyces abikoensis* TJGA-19 that have been reported to damage *Peronophythora litchii* mycelia (Xing *et al.* 2024).

In vivo *test of fungal antagonistic activity of CNXK121 culture on pitaya fruits and stems*: The ability of *Streptomyces* spp. CNXK121 to inhibit *N. dimidiatum* infection in pitaya was assessed by monitoring disease symptoms after injection of a mixture of fungal spores and actinomycete culture. After 5 days of incubation, the CNXK121 does not induce disease in pitaya (Fig. 5A-b), as the cross-section at the injection site showed no discolouration, like the negative control injected with a 1:1 mixture of PDB and Gause I medium.

In contrast, fruits injected with *N. dimidiatum* spores were covered with fungal mycelium after five days of incubation, with the cross-section showing extensive rot and necrosis, leading to browning of both the peel and flesh (Fig. 5A-c). However, treatments incorporating CNXK121 culture exhibited a progressive reduction in disease severity as the ratio of actinomycete culture to fungal spores increased. In the 1:0.5 treatment, fungal mycelium covered a portion of the fruit, causing necrosis of most of the peel and flesh (Fig.

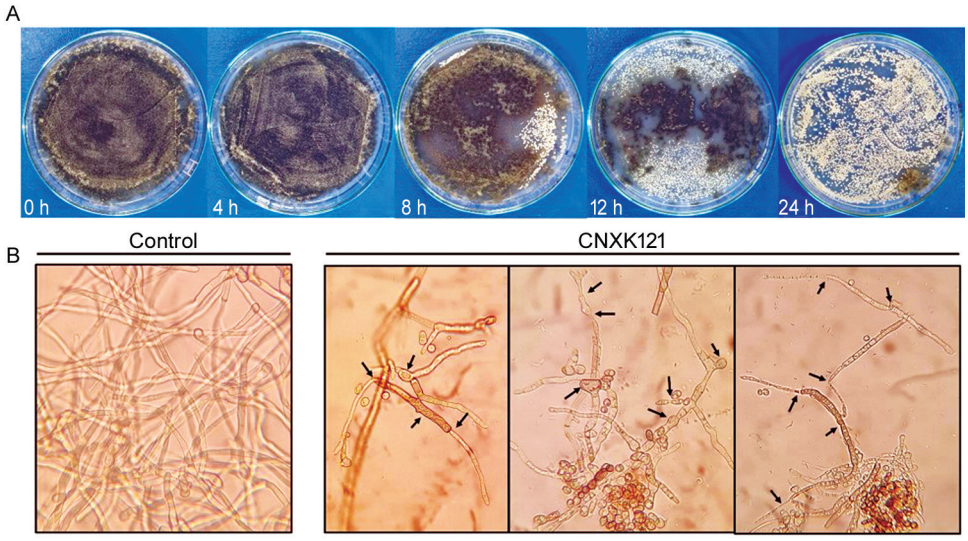

Fig. 4 Effect of actinomycete culture on fungal spore germination and mycelia. (A) Time course inhibition of *N. dimidiatum* spore germination. Black areas and light grey areas represent fungal and CNXK121 biomass, respectively; (B) Mycelial damage induced by *Streptomyces* spp. CNXK121. Black arrows indicate sites of abnormalities.

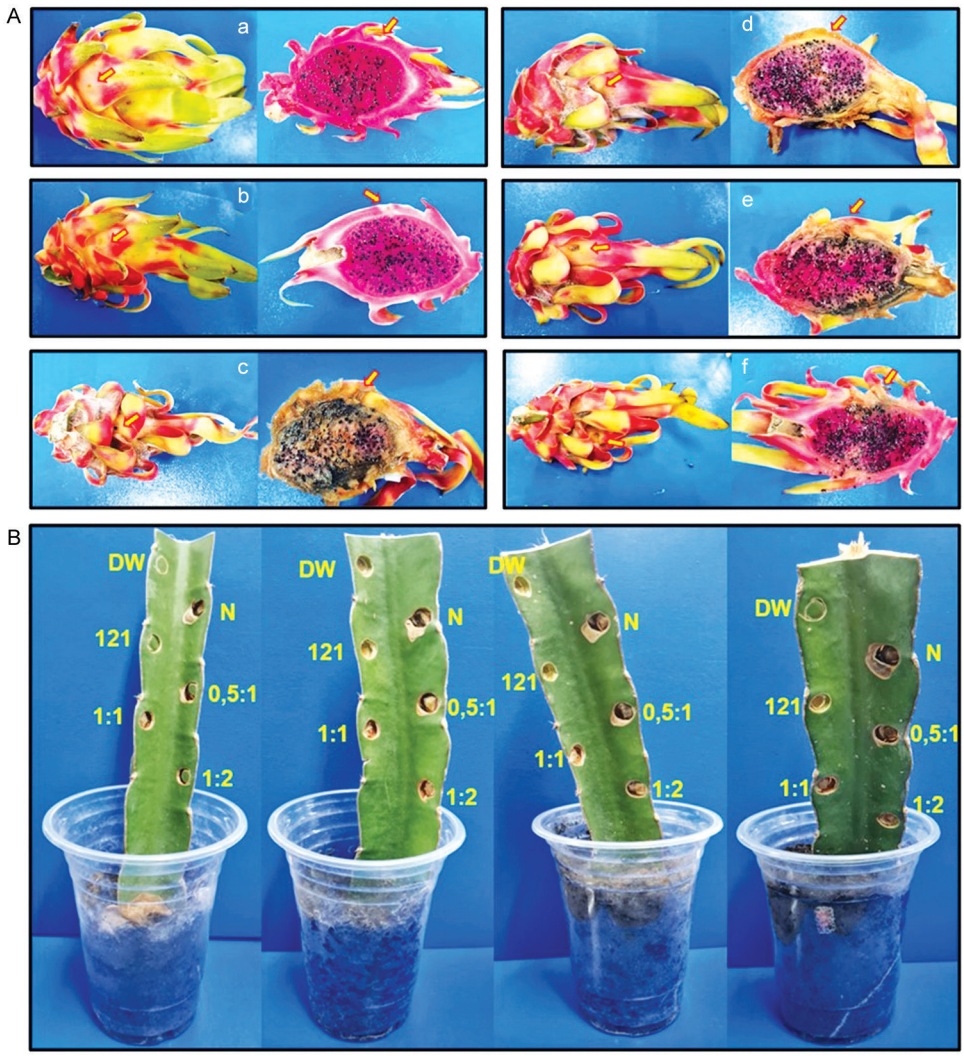

Fig. 5 Antifungal activity of *Streptomyces* spp. CNXK121 against *N. dimidiatum* on dragon fruit (A) and stem (B). (a) Negative control (PDB and Gause I medium, 1:1), (b) CNXK121 culture, (c) *N. dimidiatum* spores, (d-f) Mixtures of *N. dimidiatum* spores and CNXK121 culture at ratios of 1:0.5, 1:1 and 1:2, respectively. Red arrows indicate infection sites. DW: distilled water; 121: CNXK121 culture supernatant; N: *N. dimidiatum* spores.

5A-d). In the 1:1 treatment, the fungal growth was further restricted and tissue necrosis was reduced (Fig. 5A-e). The most significant suppression of disease symptoms was observed in the 1:2 treatment, where fungal development was minimal (Fig. 5A-f).

Additionally, the effectiveness of CNXK121 in preventing brown spot disease on stems of dragon fruit was evaluated using different treatment ratios (Fig. 5B). After 12 days of incubation, disease symptoms appeared only at sites injected with fungal spores compared to the controls. However, the severity of infection was significantly reduced at sites treated with a mixture of actinomycete culture and fungal spores, with disease suppression increased as the proportion of CNXK121 culture increased. Moreover, CNXK121 did not cause any harm to the dragon fruit plants, as the injection sites appeared similar to those treated with distilled water. These findings demonstrate that *Streptomyces* spp. CNXK121 is non-pathogenic to dragon fruit and possesses protective activity against *N. dimidiatum* on both dragon fruit stems and fruits.

The use of bacterial strains as biocontrol agents for managing plant pathogens, including those affecting pitaya, has been studied. *Bacillus subtilis* strains isolated from the phyllosphere have been studied to control rot in pitaya stems and fruits caused by the mould *Gilbertella persicaria* (Taba *et al.* 2022). Additionally, *Bacillus amyloliquefaciens* has demonstrated *in vitro* antagonism against *N. dimidiatum* (Nguyen *et al.* 2020) and actinomycetes have also been recognized for their antifungal properties against *N. dimidiatum*. Three *Streptomyces* strains (*Streptomyces rochei* UAE2, *S. antibioticus* UAE1 and *S. coelicoflavus* UAE1) have exhibited strong *in vitro* antifungal activity against *N. dimidiatum* and successfully prevented lesion formation in apples injected with the pathogen (Al Raish *et al.* 2021). *Streptomyces* spp. have also been applied in controlling stem canker disease on royal poinciana caused by *N. dimidiatum* (Al Hamad *et al.* 2021). However, research on the use of actinomycetes for the prevention of stem canker and brown spot disease in dragon fruit remains limited.

Effective pathogen management during both cultivation and post-harvest is crucial for minimising agricultural losses. This study demonstrated the effectiveness of actinomycete strains in controlling *Neoscytalidium dimidiatum*, the pathogen responsible for stem canker and fruit brown spot in pitaya in Vietnam. Among the 50 strains tested, *Streptomyces* spp. CNXK121 exhibited the strongest inhibitory effect. Its production of cell wall-degrading hydrolytic enzymes played a significant role in inhibiting fungal growth and causing structural damage to *N. dimidiatum* mycelium. Furthermore, *in vivo* tests confirmed that CNXK121, as a non-pathogenic agent, effectively suppressed stem canker and brown spot disease on pitaya. These findings suggest that *Streptomyces* spp. CNXK121 is a promising biological control agent for managing *N. dimidiatum* infections, contributing to the

development of environmentally friendly and sustainable agricultural practices.

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