BIOLOGICAL CONTROL AGAINST *Colletotrichum siamense* INFECTING MANGO BY *Bacillus* sp. TH5

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SUMMARY

In the agricultural domain, the management of anthracnose, caused predominantly by *Colletotrichum siamense*, poses a significant challenge to mango (*Mangifera indica*) production, globally recognized as a key tropical fruit commodity. Amidst this agricultural problem, *Bacillus* antagonists emerge as promising biocontrol agents, offering sustainable and environmentally friendly solutions to combat anthracnose incidence. In this study, antifungal characteristics of *Bacillus* sp. TH5 against *C. siamense* were investigated. The result revealed that the *Bacillus* sp. TH5 strain exhibited notable characteristics, including the presence of extracellular enzymes like cellulase, protease, and chitinase which contributed to its capability to degrade fungal mycelia. Moreover, even under high temperature conditions 60-90℃, the culture retained significant residual antifungal activity of about 50% after 15 minutes. Its activity also remained robust, with at least 71% efficacy after 2 hours of incubation across a broad pH range of 3.0 to 10.0. In simulated mango conditions, the strain TH5 demonstrated remarkable antagonistic efficacy, resulting in a reduction of anthracnose severity by over 95% and 69% after 4 and 6 days, respectively. These promising results underscore the potential of *Bacillus* sp. TH5 as an effective biocontrol agent against anthracnose in mango and other fruits, offering significant prospects for sustainable agricultural practices in the future.

Keywords: Anthracnose, antifungal, *Bacillus, Colletotrichum siamense*, mango.

INTRODUCTION

Anthracnose, a prevalent disease among mango trees, detrimentally affects the plant across its entire developmental spectrum, from the emergence of leaves to fruit maturation. Fungi from the *Colletotrichum* genus, notably strains of *C. gloeosporioides*, *C. capsici*, *C. falcatum*, *C. truncatum*, *C. sansevieriae*, *C. acutatum*, and *C. coccodes*, are implicated as primary anthracnose culprits affecting mango crops in India (Ajay, 2014). *C. siamense*, among five fungi identified within the *C. gloeosporioides* complex, has a broad host range, causing anthracnose in various crops worldwide, such as citrus, tea, chili, rubber, onions, and strawberries (EFSA, ECDC, 2018). Moreover, *C. siamense* and *C. asianum* have been identified as novel species causing mango anthracnose in Thailand (Rattanakreetakul *et al.*, 2023).

Conventionally, chemical fungicides are employed to combat Colletotrichum-induced anthracnose. However, their unmanageable use poses challenges, including the emergence of resistant pathogen strains and adverse health and environmental effects. Consequently, alternative biological control methods have garnered attention. Antagonistic microorganisms, such as *Bacillus mycoides* A1 and *Bacillus tequilensis* A3, *Bacillus subtilis* GYUN-2311, *Bacillus altitudinis* GS-16, have demonstrated efficacy against Colletotrichum-induced anthracnose in various crops including avocado, apple and hot pepper, and tea tree, respectively (Guerrero-Barajas *et al.*, 2020; Heo *et al.*, 2024; Wu *et al.*, 2024).

Given Vietnam's expanding mango industry and its aspirations for increased exports, which reached over 600,000 tons of mangoes valued at 310 million USD to 53 countries and territories (VNS, 2022), the search for novel biocontrol agents against mango anthracnose is of utmost importance. Therefore, the aim of this study is to characterize antagonistic activity of the strain *Bacillus* sp. TH5, isolated from soil, against *C. siamense*-induced anthracnose in mango fruits, thereby facilitating the sustainable growth of Vietnam's mango sector.

MATERIAL AND METHODS

Microbial strains and culture conditions

Four bacterial strains, TH5, TH6, TH7, TH8, isolated from mango cultivation areas in rural regions, and the fungal strain *Colletotrichum siamense* causing anthracnose disease, were preserved in the Collection of the Microbiotechnology Laboratory, Industrial University of Ho Chi Minh city, at -70℃. The bacterial strains were cultured overnight in Luria-Bertani broth (LB broth) at 37℃ with agitation at 150 rpm, while the fungal strain was cultured on Potato Glucose Agar (PGA) medium at room temperature for 5 days prior to subsequent studies.

Screening of bacteria with antifungal activity

To evaluate the antagonistic activity of bacterial strains against *C. siamense*, a co-culture method was conducted. Initially, a 5-day *C. siamense* PGA plate was prepared and cut into 5.0 mm diameter agar pieces. The fungal mycelial piece was placed in the center of a new PGA Petri dish and incubated at 30±2℃ for 2 days. Subsequently, 10 µL of bacterial inoculate were spotted 1.0 cm from the plate's edge, and antifungal effect was monitored daily as the plates were incubated at room temperature. Antifungal activity was determined by measuring the inhibition zone around the bacterial inoculated spot.

Identification of fungal pathogen and *Bacillus* antagonists

The bacterial strain which displayed highest antifungal activity were identified based on physiological, macroscopic, microscopic characteristics, and further confirmed by 16S rRNA gene amplification and sequencing using the bacterial universal primer pair: 27mF (5'-AGAGTTTGTTTGATCMTGGCTCAG-3') and 1492mR (5'-GGYTACCTTGTTACGACTT-3').

Detection of putative antifungal hydrolytic enzyme production

The biosynthesis of putative enzymes involved in the hydrolysis of the mycelium cells such as chitinase, protease, and cellulase was determined by culturing bacterial strains on LB agar supplemented with the respective substrates such as chitin, casein, and carboxymethyl cellulose (10 g/L). Enzymatic activity was detected via the substrate degradation zone after 3 days of incubation at 37℃. Lugol reagent was used to detect chitinase and cellulase activity while protease activity was observed with trichloroacetic acid 10%.

Assay for hyphal growth inhibition

To examine the influence of bacterial strain TH5 on the fungal mycelia, a 5-day static co-incubation was conducted. The bacteria were initially cultured in LB medium with agitation at 150 rpm at 37℃ until the OD_{600nm} reached 0.6. The bacterial culture was then centrifuged for 15 minutes at 4000 rpm, and the supernatant was filtered using a 0.45 µm membrane filter. Concurrently, the fungal mycelium was grown in potato glucose broth for 5 days. The fungal mycelium was harvested and exposed to the filtered bacterial culture that cultured in LB broth at 37℃ for 24 hours. Sterilized LB medium was used as a control. The fungal inhibitory effects were analyzed microscopically by comparing the treated samples with the control.

Impact of temperature and pH on antagonistic activity of bacterial culture

Bacterial strain TH5 was cultured in LB broth at 37℃ for 24 hours and collected cell-free bacterial supernatant by centrifuge at 13000 rpm, 4℃ for 10 min. The thermal stability of TH5 culture supernatant were assessed by incubation at high temperature of 60-90℃ for 5, 10 and15 minutes. Similarly, for the pH stability assessment, the cultures were adjusted to pH values ranging from 3.0 to 10.0 and incubated at room temperature for 2 hours. The residual antifungal activity following these treatments was measured using an agar-well diffusion assay and expressed as a percentage compared to the untreated culture.

Disease suppression assay

Mango fruit models were employed to evaluate the biological control effect of bacterial strain TH5. The uninjured and healthy mangoes were washed with sterile distilled water three times, treated with a 100 ppm chlorine solution, then rinsed again with sterile distilled water, and disinfected with alcohol 70% as the final step. *C. siamense* mycelia were prepared by growing them in PGB medium at room temperature for 5 days, while the bacterial culture in LB medium was prepared after 16 hours of growth at 37℃. The bacterial culture and mycelium suspension were mixed in a 1:1 ratio, with 10 µL of this mixture being injected into the mango fruit (experimental sample). The same treatment was done for the positive control sample with only the mycelium suspension and the negative control sample with only sterilized LB medium. The mangoes were incubated in a sterile humidified chamber for 6 days. The development of disease symptoms was recorded and quantified based on the lesion surface area using QuPath v0.5.1 software.

Data analysis

The results of the experiments are an average of 3 replicates. The data were calculated and graphed on Microsoft Excel 2013 and ANOVA statistical analyzed by Statgraphics Centurion 18 software.

RESULTS AND DISCUSSION

Antifungal activity of *Bacillus* strains against *Colletotrichum siamense*

The ability of four different bacterial strains to inhibit *C. siamense*, which causes anthracnose in mango, after 3 and 5 days of co-culture is shown in Figure 1. After only 3 days of incubation, the TH5 strain demonstrated a clear inhibitory effect on the fungal hyphae with a distinct inhibition zone. In contrast, the TH8 strain showed less clear inhibition, and no inhibition were observed in the case of TH6 and TH7 strains (Figure 1A).

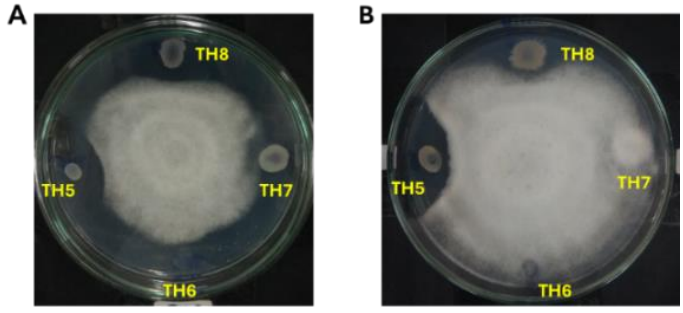

Figure 1. Antagonistic ability of *Bacillus* spp. after 3 days (A) and 5 days (B) of co-culture

The inhibitory effects of the bacterial strains were more pronounced after 5 days of incubation (Figure 1B). While the TH6 and TH7 strains were covered by the fungal mycelia, the TH5 and TH8 strains exhibited a complete inhibition zone around the bacterial inoculated spots, measuring 6.67 ± 0.72 mm and 6.0 ± 0.47 mm, respectively. Therefore, the TH5 bacterial strain with strongest antagonistic activity was selected for further studies, including in trials to inhibit this fungal strain in post-harvest mango preservation.

Identification of the bacterial strain TH5

Regarding macroscopic morphology, when cultured in LB plate at pH 7.0 and 37℃, after 48 hours, the TH5 strain formed colonies measuring 2-3 mm in diameter, with an opaque white color, raised surface, irregular edges, and a characteristic wrinkled membrane as shown in Figure 2Aa. Gram staining and endospore staining results, depicted in Figure 2Ab and Ac, indicate that the TH5 strain is a Gram-positive bacterium capable of producing endospores, which tend to be positioned asymmetrically. The catalase production test revealed that upon dissolving the colony in a drop of 3% H_2O_2, bubbles were produced suggesting that this is a catalase producing strain (data not shown). Additionally, the 16S rRNA gene of this strain was sequenced. BLAST comparison of this sequence (1446 bp) with the GenBank database showed a 99.93% similarity to *Bacillus velezensis* Bac57 (CP033054.1). A phylogenetic tree with six taxa was constructed using Mega5 software with the UPGMA method and 1000 bootstrap replicates. The results, presented in Figure 2B, show that the TH5 strain clusters with *Bacillus velezensis* Bac57 with a bootstrap value of 90%, confirming that the TH5 strain is closely related to *Bacillus velezensis* and has been designated as *Bacillus* sp. TH5.

Bacillus velezensis has been recognized as a potential research strain due to its ability to synthesize various novel lipopeptides with surfactant properties and high antimicrobial activity (Ruiz-Garcia *et al.*, 2005). In addition, the B. velezensis G341 strain isolated by Lim et al. (2017) from 4-year-old ginseng was proved to synthesize both volatile and soluble compounds, inhibiting *Botrytis cinerea*, the cause of gray mold in tomatoes, and Rhizoctonia solani, the cause of sheath blight in rice (Lim *et al.*, 2017).

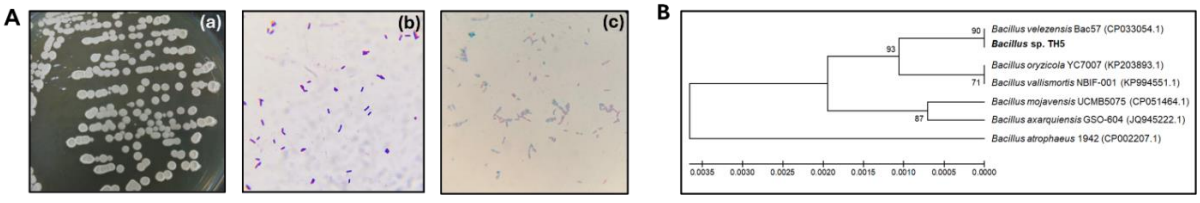

Figure 2. Identification of *Bacillus* sp. TH5. (A-a) Colony morphology, (A-b) Gram staining, (A-c) Endospore staining and (B) Phylogenetic tree based on the 16S rRNA sequence region

***Bacillus* sp. TH5 production of enzymes related to cell wall degradation**

In order to further explore the factors affecting the antifungal activity of *Bacillus* sp. TH5, the biosynthesis of extracellular chitinase, protease and cellulase was investigated on culture medium corresponding substrates. The presence of hydrolytic enzymes after 3 days of incubation is shown in Figure 3. *Bacillus* sp. TH5 produced strong protease, cellulase and chitinase activity with zone of substrate hydrolysis of 3.6 ± 0.2 cm, 1.8 ± 0.2 cm and 3.4 ± 0.1 cm respectively. Compared to other studies, the extracellular protease from *Bacillus* sp. TH5 exhibited higher activity than that from *Paenibacillus polymyxa* APEC128, which possesses antifungal activity against *C. gloeosporioides* and *C. acutatum* (Kim *et al.*, 2016). Biosynthesis of hydrolytic enzyme chitinase and protease of *Bacillus* sp. TH5 are therefore consistent with the observed growth inhibition of *C. siamense.*

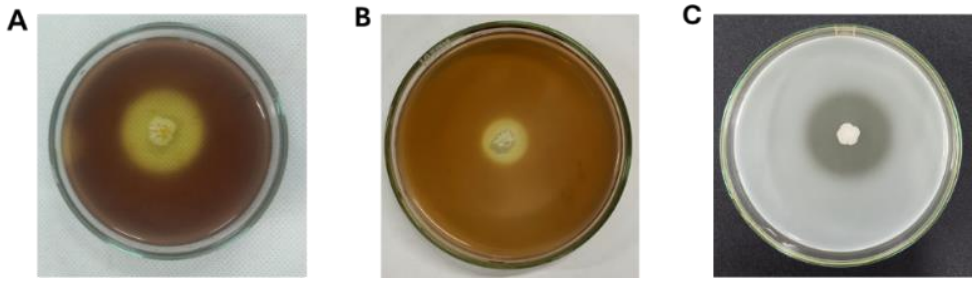

Figure 3. Biosynthesis of (A) chitinase, (B) cellulase and (C) protease of *Bacillus* sp. TH5

Effect of *Bacillus* sp. TH5 culture on the mycelia of *C. siamense*

The results showed that the mycelia in the control test were uniform and healthy, with intact cell walls and normal hyphal tips (Figure 4A). In contrast, mycelial samples incubated with bacterial culture exhibited abnormal structures, including heterogeneous thickness, delicate and thin mycelium, and several signs of shrinkage, swelling, and even disruption (Figure 4B-D). These effects are likely attributed to the hydrolytic action of extracellular protease and particularly chitinase produced by *Bacillus* sp. TH5. Similar findings regarding *Colletotrichum* mycelial damage have been reported in previous studies (Ashwini, Srividya, 2014; Nawaz *et al.*, 2018).

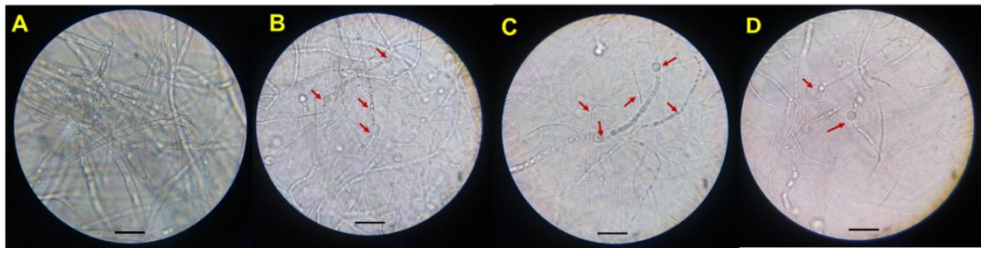

Figure 4. Antifungal effect of *Bacillus* sp. TH5 culture on fungal mycelia. (A) Healthy fungal mycelium in PGB; (B, C, D) Mixture of fungal mycelia and bacterial culture; arrows: damaged sites; scale bar: 10 µm

Temperature and pH stability of antagonistic *Bacillus* sp. TH5 culture

Figure 5A illustrates the temperature stability of the antagonistic *Bacillus* sp. TH5 culture across different temperature levels. The residual antifungal activity ranged from 66.67% to 73.33% with no significant difference observed when bacterial cultures were treated for 5 minutes at 60-90℃ (ANOVA, n = 3, $p < 0.05$). Although there was no significant difference for the 10-minute and 15-minute treatments (ANOVA, n = 3, $p < 0.05$), the inhibitory activity slightly decreased with increasing treatment time, showing 53.33-66.67% and 46.67-53.33% residual activity for the 10-minute and 15-minute treatments, respectively. The maintenance of at least 50% antifungal activity after up to 15 minutes of heat treatment indicates the heat stability of the cell-free bacterial culture of *Bacillus* sp. TH5.The thermostability of antifungal activity in cell-free cultures has also been observed in the study of Nawaz et al. (2018). Accordingly, partially purified biosurfactants from *B. licheniformis* OE-04 displayed increased antagonistic activity against *C. gossypii* after treatment at 28-100℃ (Nawaz *et al.*, 2018). The culture filtrate of *B. subtilis* B-FS06 was shown to maintain antagonistic activity against *Aspergillus flavus* after treatment at 20-100℃ for 30 minutes (Zhang *et al.*, 2008). Taken altogether, our results suggest that the culture supernatant of *Bacillus* sp. TH5 contains different components contributing to anti-*C. siamense* activity, with varying thermal sensitivities under different temperature conditions and exposure times.

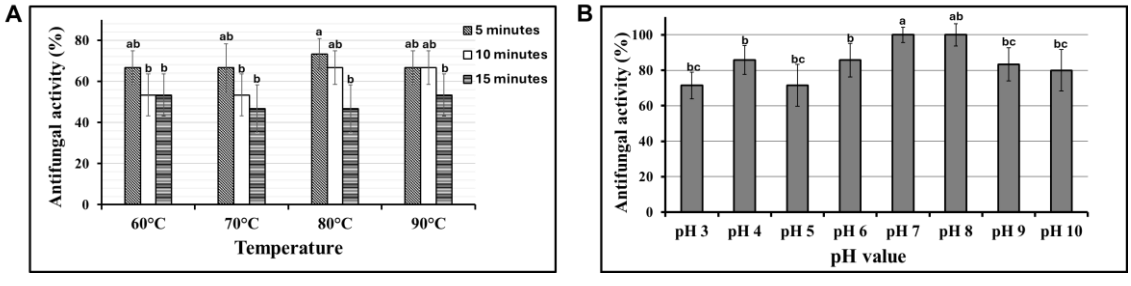

Figure 5. (A) Thermal stability and (B) pH stability of bacterial culture on the inhibition of *C. siamense*.

[a,b] Different letters indicate statistically significant differences ($p<0.05$)

The data presented in Figure 5B demonstrate that the bacterial culture maintained robust antifungal activity over a wide pH range of 3.0-10.0, with residual activity between 71% and 100%. The antifungal activity was fully stable at pH levels of 7.0 and 8.0, while at other pH levels, it decreased by only about 20% with no statistical difference (ANOVA, n = 3, $p < 0.05$). Similar pH stability has been observed in another study. For instance, *B. licheniformis* OE-04 surfactants maintained stability against *C. gossypii* over the same pH range (Nawaz *et al.*, 2018). These findings indicate that the culture supernatant of *Bacillus* sp. TH5 is pH-stable across a broad spectrum, suggesting its potential for use in both acidic and basic environments.

***C. siamense* anthracnose disease suppression assay on mango model**

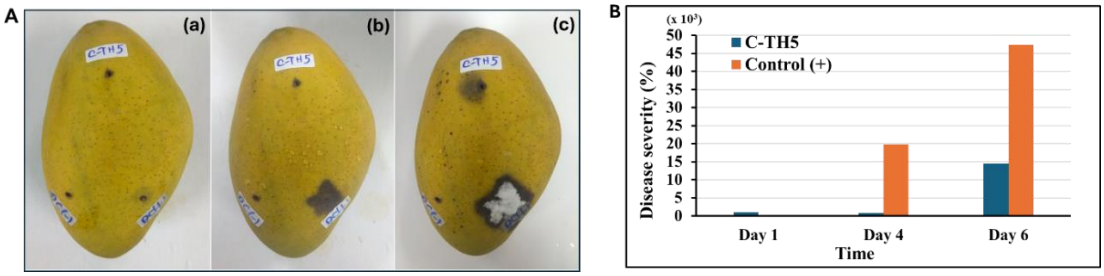

Figure 6. Disease expression of *C. siamense* in mango model (A) after 1 day (Aa), 4 days (Ab), 6 days (Ac) and the disease severity displayed in percentage (B). DC (-): sterilized LB medium; DC (+): *C. siamense* suspension; (C) C-TH5: mixture of *C. siamense* and *Bacillus* sp. TH5

Disease symptoms, including color changes and soft rot, were monitored on mango fruit after 1, 4, and 6 days of treatment (Figure 6). After the first day, there was minimal difference in disease severity between the samples injected with the mold (Figure 6Aa). However, by the fourth and sixth days, the disease symptoms caused by *C. siamense* were pronounced (Figure 6Ab-c). In the positive control sample, lesion area expanded significantly, by 1984.66% and 4735.48%, compared to the negative control after 4 and 6 days of incubation, respectively. Additionally, severe lesions with extensive fungal mycelial growth and decay were observed in the positive control sample after 6 days. In contrast, the test samples treated with a mixture of *C. siamense* and *Bacillus* sp. TH5 showed lesion area expansions of only 87.90% and 1454.75% after 4 and 6 days, respectively. This indicates that *Bacillus* sp. TH5 reduced disease severity in mango fruit caused by *C. siamense* by 95.6% and 69.3% after 4 and 6 days, respectively (Figure 6B). In related post-harvest studies, *B. amyloliquefaciens* PMB04 strains was also shown to reduce *C. gloeosporioides* anthracnose severity by 15-37.5%, *Stenotrophomonas rhizophila* decreased disease severity by 85%, and *Torulaspora indica* DMKU-RP35 along with *Pseudomonas aspenensis* DMKU-SP67 reduced disease severity by 82.4-93.9% (Konsue *et al.*, 2020; Liang *et al.*, 2022; Reyes-Perez *et al.*, 2019). Overall, these findings suggest that *Bacillus* sp. TH5 is a promising biological control agent for managing anthracnose caused by *C. siamense* on mango fruit.

CONCLUSION

Vietnam is one of the top mango-exporting countries in the world. Anthracnose disease, caused by *Colletotrichum siamense*, poses significant economic losses to the mango cultivation and trade industry in Vietnam and globally. In this present study, the bacterial strain *Bacillus* sp. TH5, isolated from mango-growing regions, demonstrated strong antagonistic properties against *C. siamense*. The antifungal activity of the culture supernatant of *Bacillus* sp. TH5 was shown to be stable across a wide range of temperatures and pH levels. With its ability to damage *C. siamense* mycelium and inhibit anthracnose symptoms when applied in mango preservation, *Bacillus* sp. TH5 has the potential to contribute significantly to the development of environmentally friendly biological control methods for managing anthracnose in agriculture.

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KIỂM SOÁT SINH HỌC *Colletotrichum siamense* GÂY THÁN THƯ Ở XOÀI BỞI *Bacillus* sp. TH5

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TÓM TẮT

Trong nông nghiệp, kiểm soát bệnh thán thư, do *Colletotrichum siamense* gây ra, là thách thức đối với quy trình sản xuất xoài (*Mangifera indica*). Vi khuẩn *Bacillus* được xem như tác nhân kiểm soát sinh học đầy hứa hẹn, giải pháp bền vững và thân thiện với môi trường để chống lại bệnh thán thư trong nông nghiệp. Trong nghiên cứu này, đặc tính kháng nấm của *Bacillus* sp. TH5 chống lại *C. siamense* đã được nghiên cứu. Kết quả cho thấy *Bacillus* sp. TH5 thể hiện những đặc điểm đáng chú ý, bao gồm sự hiện diện của các enzyme ngoại bào như cellulase, protease và chitinase góp phần làm tổn thương hệ sợi nấm *C. siamense*. Hơn nữa, ngay cả trong điều kiện nhiệt độ cao 60°C-90°C, dịch nuôi cấy *Bacillus* sp. TH5 vẫn giữ được hoạt tính kháng nấm khoảng 50% sau 15 phút. Hoạt tính kháng nấm vẫn duy trì với 71% sau 2 giờ ủ trong khoảng pH từ 3,0-10,0. Trong mô hình quả xoài, chủng *Bacillus* sp. TH5 cho thấy hiệu quả đối kháng vượt trội với việc giảm mức độ gây bệnh thán thư lần lượt trên 95% và 69% sau 4 và 6 ngày. Những kết quả đầy hứa hẹn này nhấn mạnh tiềm năng của *Bacillus* sp. TH5 như một tác nhân kiểm soát sinh học hiệu quả chống lại bệnh thán thư ở xoài và các loại trái cây khác, mang lại triển vọng đáng kể cho các hoạt động nông nghiệp bền vững trong tương lai.

Từ khóa: Bệnh thán thư, thuốc kháng nấm, *Bacillus, Colletotrichum siamense*, xoài.

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