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Biocontrol effectiveness of *Trichoderma asperelloides* SKRU-01 and *Trichoderma asperellum* NST-009 on postharvest anthracnose in chili pepper

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ABSTRACT

This study assessed the effectiveness of *Trichoderma asperelloides* SKRU-01 and *Trichoderma asperellum* NST-009 in controlling postharvest anthracnose in chili fruits caused by *Colletotrichum gloeosporioides* PSU-03, using both *in vitro* assays and studies on chili fruits. *In vitro* findings indicated that both SKRU-01 and NST-009 exhibited inhibitory effects on PSU-03 through synergistic mechanisms, involving competition for space and nutrients, as well as the production of non-volatile and volatile compounds. Culture filtrates of both strains, at low concentrations (diluted at 1/1000), displayed a robust antifungal effect on PSU-03, achieving over 50% inhibition. This efficacy, however, was lower than that achieved by five chemical fungicides (propiconazole®, prochloraz®, metalaxyl®, azoxystrobin®, and thiram®). The efficacy against anthracnose of SKRU-01 (60.34% protection) and NST-009 (62.07% protection) was significantly higher than metalaxyl®, azoxystrobin®, and thiram®, but lower than propiconazole® and prochloraz®. The two strains demonstrated both preventive and curative effects. Furthermore, results from tests on chili seed germination showed that culture filtrates SKRU-01 significantly inhibited the germination of chili pepper seeds and the growth of seedlings in lab settings. Overall, this study suggests that both SKRU-01 and NST-009 hold the potential for managing postharvest anthracnose in chili fruits caused by *C. gloeosporioides*. It is crucial to note, however, that culture filtrates SKRU-01 should not be used for biostimulating root development in chili peppers. This research provides valuable insights into the potential application of these biocontrol agents, contributing to enhancing the quality and shelf life of chili produce.

1. Introduction

Anthracnose, an economically significant disease affecting various vegetables, including chili peppers, is caused by the *Colletotrichum* fungus species (Bhakat et al., 2023; Kwon et al., 2022; Suprapta, 2022). The disease in Thailand is attributed to four *Colletotrichum* species: *C. truncatum* (alternatively known as *C. capsici*), *C. acutatum*, *C. gloeosporioides*, and *C. siamense* (Suwannarat et al., 2017). These diseases are serious and common postharvest illnesses that have a huge effect on the fruit's marketability (Nantawanit et al., 2010). Specifically, in certain developing countries such as Thailand, Korea, Indonesia, and Malaysia, the presence of *Colletotrichum* species has been reported to result in a reduction of marketable yield by 15% in Korea, 35% in Indonesia, 50% in Malaysia, and an approximately 80% yield loss in Thailand (Poonpolgul & Kumphai, 2007; Ridzuan et al., 2018). Typically, employing chemical methods is a widely used approach to manage postharvest diseases in chili fruit. Nevertheless, utilizing chemical fungicides for controlling chili anthracnose pathogens may lead to the emergence of fungicide-resistant strains (Kim et al., 2023), posing potential risks to both consumers and the environment (Chen & Ying, 2015; Kim et al., 2023; Zubrod et al., 2019).

The use of microbial antagonistic agents for biological control is widely recognized as a promising alternative to chemical fungicides for the management of postharvest diseases (Boro et al., 2022; de Sousa & Granada, 2023; Fenta et al., 2023; Lahlali et al., 2022). Numerous researchers have investigated effective strategies for controlling vegetable diseases, focusing on novel biocontrol agents such as microbes like *Bacillus* (Guo et al., 2023; Li et al., 2023; Shao et al., 2023), *Pseudomonas*

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(Ahmed et al., 2023; Mehmood et al., 2023), *Streptomyces* (Boukaew et al., 2018, 2021), and *Trichoderma* (Boukaew et al., 2023, 2024a, 2024b; Nujthet et al., 2023). *Trichoderma* stands out among these, being widely acknowledged as a promising biocontrol agent that has undergone extensive evaluation and global implementation (Ren et al., 2022; Woo et al., 2014). For example, *T. harzianum* (Amira et al., 2017; Güçlü & Özer, 2022; Verma et al., 2023), *T. asperellum* (Li et al., 2018), *T. virens* (Gan et al., 2022; Tomah et al., 2023), *T. viride* (Gao et al., 2023; Verma et al., 2023), *T. atroviride* (Chilosi et al., 2020), and *T. asperelloides* (Boukaew et al., 2023, 2024a, 2024b). These widely recognized antagonistic fungi have found commercial applications in the control of various diseases (Abo-Elyousr et al., 2014; Alvindia, 2018). They are recognized for exerting highly antagonistic activities against plant pathogens through diverse mechanisms, including competition for natural resources, antibiosis, mycoparasitism, and induction of plant disease resistance. Additionally, they promote root development and enhance overall plant growth (Amira et al., 2017; Lorito et al., 2010; Qualhato et al., 2013; Vinale et al., 2014).

Several studies have reported successful utilization of *Trichoderma* for controlling chili anthracnose caused by *Colletotrichum* species (Kim et al., 2023; Nujthet et al., 2023; Ruangwong et al., 2023; Yadav et al., 2021). For instance, Yadav et al. (2021) found that chili seeds treated with a combination of *T. asperellum* and *T. harzianum* reduced the development of anthracnose caused by *C. truncatum* in fruits by 78.67%. In another study, Nujthet et al. (2023) reported that chili fruits treated with *T. asperellum* KUFA 0111 (0.85 cm disease incidence) and *T. harzianum* KUFA 0141 (1.02 cm disease incidence) showed a reduction in anthracnose incidence caused by *C. truncatum* compared to the control (2.21 cm disease incidence). Furthermore, chili pepper fruit treated with *T. koningiopsis* PSU3-2 exhibited a complete (100%) reduction in anthracnose development caused by *C. gloeosporioides* (Ruangwong et al., 2023). In particular, *T. asperelloides* SKRU-01 has demonstrated notable efficacy in inhibiting the growth of food spoilage fungi in peanuts, particularly those caused by *Aspergillus parasiticus* and *A. flavus* (Boukaew et al., 2023, 2024a, 2024b). However, it has never been tested against *Colletotrichum* species. *T. asperellum* NST-009, a locally sourced fungus isolated from forest soil in Nakhon Si Thammarat, Thailand, has undergone development as a biological product. Demonstrating effectiveness, it has been successful in controlling *Phytophthora* leaf fall in rubber trees (Promwee et al., 2017), addressing white root disease in rubber trees (*Hevea brasiliensis*) (Promwee et al., 2022), and mitigating *Cercospora* leaf spot (Promwee & Intana, 2022). Currently recommended by the Agricultural Microbial Production and Service Center at Walailak University, it is being distributed to farmers across Thai provinces for the control of plant diseases and promotion of growth in various crops, including rice, durian, oil palm, and vegetables, thus serving as a positive control.

While some *Trichoderma* species are widely employed to control anthracnose disease, to the best of our knowledge, *T. asperelloides* has not been tested against *Colletotrichum*, and this area remains largely unexplored. Therefore, in this study, we evaluated the effectiveness of both strains SKRU-01 and NST-009 in the management of postharvest anthracnose in chili fruits induced by *C. gloeosporioides*. This assessment includes both *in vitro* assays and investigations conducted on actual chili fruits. Additionally, the study explores the impact of antifungal metabolites from both strains SKRU-01 and NST-009 on biostimulating root development in chili peppers.

2. Material and methods

2.1. Sources of materials

2.1.1. Fungal materials and inoculum preparation

Trichoderma asperelloides SKRU-01, as previously documented (Boukaew et al., 2023), was isolated from loam soil samples. *Trichoderma asperellum* NST-009 was sourced from the microbial culture collection at the Agricultural Microbial Production and Service Center, Walailak University, Thailand. Both strains were cultivated on potato dextrose agar (PDA) at 30 °C for 7 d. The spore inoculum was prepared by suspending it in sterile 0.1% Tween 80. The spore count was conducted using a hemacytometer before being diluted to achieve the required concentration with sterile distilled water.

Colletotrichum gloeosporioides PSU-03, identified as the most aggressive strain causing anthracnose in chili fruit, was isolated from chili fruit infected with postharvest anthracnose (Boukaew et al., 2021). The fungal isolate was cultivated on PDA plates at 30 °C for 12 d. The spore inoculum preparation of PSU-03 followed the procedure described above.

2.1.2. Commercial chemical fungicides

Five chemical fungicides were utilized in this study: 45% (w/v) prochloraz® (AG-GRO (Thailand) Co., Ltd.), 25% (w/v) metalaxyl® (AG-GRO (Thailand) Co., Ltd.), 25% (w/v) azoxystrobin® (SAIMA CHEMICAL CO., Ltd.), 25% (w/v) propiconazole® (SAIMA CHEMICAL CO., Ltd.), and 80% (wg) thiram® (Hebei Tangun Biotech Co., Ltd.).

2.1.3. Chili fruit and seed chili material

The chili pepper cultivar 'Orange T2005′ was used in this study. Red chili fruits were obtained from the Hatyai market in Songkhla province, ensuring uniform sizes (12–19 g per fruit, 2.65 × 12.63 cm, W × L). The chili fruits were selected devoid of any disease symptoms or visible wounds. Upon harvesting, the samples were promptly utilized in the experiments or stored at 20 °C under a relative humidity of 92–94% for up to 48 h, following the approach established by Maurya et al. (2020). To prepare the fruits for testing, a surface sterilization process was performed. The chilis were immersed in a sodium hypochlorite solution with a 3% (v/v) concentration for 3 min. Afterward, several rinses with sterile distilled water were conducted (Abd-Alla, 2005). Subsequently, the chilis underwent aseptic drying for 30 min within a laminar airflow chamber.

2.2. In vitro evaluation of the mycelial growth of strain PSU-03 using both strains SKRU-01 and NST-009

2.2.1. Dual culture

The antagonistic interaction between strain SKRU-01 or strain NST-009 and the PSU-03 strain was assessed using a dual culture assay. The experimental procedures for antifungal activity followed the methods previously outlined by Boukaew et al. (2023). Daily monitoring of the radial growth of each mycelial colony was conducted. For each treatment, three replicates were performed. The percentage of mycelial growth inhibition (%) by strain SKRU-01 or strain NST-009 was determined following the method described by Parizi et al. (2012). This calculation involved the formula: Percent inhibition of growth = $(R_1–R_2/R_1) \times 100$, where R_1 represents the radial mycelial growth in the control treatment, and R_2 represents the radial mycelial growth in the SKRU-01 or NST-009 strain treatment.

2.2.2. Culture filtrates effect

For the preparation of culture filtrates, each strain SKRU-01 and strain NST-009 was grown in potato dextrose broth (PDB) at 30 °C for 10 d. The mycelial mats were removed by filtration through Whatman No. 1 filter paper to obtain a clear culture filtrate, which was then filter-sterilized through a 0.45 μm Millipore membrane (Sartorius®) to yield the culture filtrates (referred to as culture filtrates SKRU-01 or NST-009). The effects of culture filtrates SKRU-01 or NST-009 were tested under different conditions, including undiluted culture filtrates, 1/10 diluted culture filtrates, 1/100 diluted culture filtrates, 1/1000 diluted culture filtrates, and autoclaved culture filtrates at 121 °C for 15 min, against PSU-03 strain, comparing them to five chemical fungicides—-prochloraz® (45% (w/v)), metalaxyl® (25% (w/v)), azoxystrobin® (25% (w/v)), propiconazole® (25% (w/v)), and thiram® (80% (wg)).

One mL of each antifungal agent was combined with 9 mL of melted sterile PDA and poured onto a 9 cm diameter culture plate. Sterile distilled water mixed with 9 mL of melted sterile PDA at an equivalent amount was used as a control. A 5 mm diameter plug of mycelia was excised from a 3-day-old strain PSU-03 colony and transferred onto the center of the test agar plates. The cultures were then incubated at 30 °C. After seven days of incubation, the diameters of the colonies of strain PSU-03 were measured, and the percentage inhibition of hyphal growth was calculated as described previously. Three replicates were conducted for each treatment.

2.2.3. Volatile organic compounds effect

The impact of volatile organic compounds (VOCs) generated by both strains SKRU-01 and NST-009 against the PSU-03 strain was examined. The preparation of VOCs followed the procedure previously detailed by Boukaew, Petlamul, Yossan, et al. (2024). The dual-culture sealed plate technique (van Zijll de Jong et al., 2023) was employed to assess the *in vitro* antifungal activity of strain SKRU-01 or NST-009 VOCs against the PSU-03 strain. A 5 mm diameter plug of mycelia was excised from a 3-day-old strain PSU-03 colony and transferred onto the center of PDA dishes. Simultaneously, PDA plates of strain SKRU-01 or NST-009 VOCs were prepared, and the lid of the plates was replaced by a base plate of PDA for the pathogen. The control group was inoculated with the fungal pathogen only. The two base plates were sealed with Parafilm and incubated at 30 °C. After seven days of incubation, the diameters of the colonies of strain PSU-03 were recorded, and the percentage inhibition of hyphal growth was calculated as described above. Three replicates were conducted for each treatment.

2.3. Biocontrol potential of both strains SKRU-01 and NST-009 against strain PSU-03 on chili fruits

Preliminary findings indicated that concentrations lower than 10^5 spores/mL of both SKRU-01 and NST-009 strains were ineffective in controlling postharvest anthracnose in chili fruits caused by strain PSU-03. Therefore, for this study, we selected a concentration of 10^5 spores/mL for both strains to effectively control postharvest anthracnose caused by strain PSU-03. Each experimental treatment involved eight chili fruits, each with two wounds measuring 1 mm in diameter and approximately 0.5 mm in depth. A PSU-03 strain spore suspension containing 1×10^4 spores/mL, amounting to 5 μL, was meticulously applied to each wound. After allowing for an air-drying period of around 4 h, the wounds were subsequently inoculated with 10 μL of each respective biocontrol treatment. The biocontrol treatments were classified as follows: (A) Culture filtrates SKRU-01; (B) Autoclaved cultures SKRU-01; (C) SKRU-01 spore concentration of 1×10^5 spores/mL; (D) Culture filtrates NST-009; (E) Autoclaved culture filtrates NST-009; (F) NST-009 spore concentration of 1×10^5 spores/mL; (G) Propiconazole® (25% (w/v)); (H) Prochloraz® (45% (w/v)); (I) Metalaxyl® (25% (w/v)), (J) Azoxystrobin® (25% (w/v)); (K) Thiram® (80% (wg)). The control group was treated by applying sterile distilled water. The treated chili fruits were enclosed in plastic containers lined with polyethylene to maintain high humidity levels (approximately 85%) and were then placed in an incubation environment set at 30 °C. After 10 days of inoculation, the size of the anthracnose symptoms in each chili fruit was measured with a vernier caliper. The formula used to calculate the percentage of protection of chili fruit by the antifungal agent was as follows: Percent protection of chili fruit = ((Size of anthracnose symptoms of control - Size of anthracnose symptoms of treated)/Size of anthracnose symptoms of control) × 100. Each treatment consisted of eight chili fruits, and the experiment was replicated twice.

2.4. Impact of varying SKRU-01 and NST-009 spore concentrations against PSU-03 strain on chili fruits

Each experimental treatment involved eight chili fruits, each featuring two wounds as previously described. A PSU-03 strain spore suspension containing 1×10^4 spores/mL, totaling 5 μL, was meticulously applied to each wound. After allowing for an air-drying period of approximately 4 h, the wounds were subsequently inoculated with 10 μL of the respective biocontrol treatment. The biocontrol treatments were categorized as follows: (A) SKRU-01 strain at a spore concentration of 1×10^5 spores/mL; (B) SKRU-01 strain at a spore concentration of 1×10^6 spores/mL; (C) SKRU-01 strain at a spore concentration of 1×10^7 spores/mL; (D) SKRU-01 strain at a spore concentration of 1×10^8 spores/mL; (E) NST-009 strain at a spore concentration of 1×10^5 spores/mL; (F) NST-009 strain at a spore concentration of 1×10^6 spores/mL; (G) NST-009 strain at a spore concentration of 1×10^7 spores/mL; and (H) NST-009 strain at a spore concentration of 1×10^8 spores/mL. The control group is treated by applying sterile distilled water. The treated chili fruits were enclosed in plastic containers lined with polyethylene to maintain a high-humidity environment (approximately 85%) and were then placed in an incubation setting at 30 °C. Ten days post-inoculation, the size of disease symptoms in each fruit was measured using a vernier caliper. Each treatment consisted of eight chili fruits, and the experiment was replicated twice.

2.5. Preventive and curative efficacy of both strains SKRU-01 and NST-009 against PSU-03 strain on chili fruits

To evaluate the potential preventive impact of both strains SKRU-01 and NST-009 against PSU-03 on chili fruits, each pruning wound was treated with a 10 μL spore suspension of either strain SKRU-01 or strain NST-009 at a concentration of 1×10^5 spores/mL. Subsequently, the wound was inoculated with a 5 μL spore suspension of the PSU-03 strain at a concentration of 1×10^4 spores/mL. The inoculation of PSU-03 was performed at time intervals of 0, 2, 4, or 6 h following the application of strains SKRU-01 or NST-009. The control group was treated by applying sterile distilled water.

To explore the potential curative effect of both strains SKRU-01 and NST-009 against the PSU-03 strain, the same procedure was followed, except that inoculation of PSU-03 at a concentration of 1×10^4 spores/mL occurred before treatment. In this case, a spore suspension of either strain SKRU-01 or strain NST-009 at a concentration of 1×10^5 spores/mL was applied after PSU-03 inoculation. The treatment with either strain SKRU-01 or strain NST-009 was conducted at intervals of 0, 2, 4, or 6 h following PSU-03 strain inoculation. The control group was treated by applying sterile distilled water.

All treated fruits were enclosed within polyethylene-lined plastic containers to ensure a heightened humidity level (approximately 85%), and they were placed in an incubation environment set at 30 °C. Ten days post-inoculation, the size of disease symptoms in each fruit was measured using a vernier caliper. Each treatment consisted of eight chili fruits, and the experiment was replicated twice.

2.6. The effects of culture filtrates SKRU-01 and NST-009 on chili seed germination

The seeds were subjected to a 3-min immersion in a sodium hypochlorite solution with a concentration of 3% (v/v). Subsequently, they underwent multiple rinses using sterile distilled water, following the methodology established by Abd-Alla (2005). After these steps, the seeds were aseptically dried for 30 min within a laminar airflow chamber.

The impact of culture filtrates SKRU-01 and NST-009, previously shown to inhibit the growth of strain PSU-03, was evaluated for its potential effect on seed germination. To conduct the experiment, two hundred and seventy chili seeds were soaked in 5.0 mL of culture filtrates from either SKRU-01 or NST-009 for 30 min. The control group is treated by applying sterile distilled water. The seeds were then dried using aseptic procedures in a laminar airflow chamber for an additional 30 min. Following the drying process, the chili seeds were placed in a

moisture-controlled plastic box (measuring 17.7 × 25.1 × 9.2 cm in width, length, and height, respectively). The assessment of seed germination toxicity included the measurement of percentages for seed germination, stem length, root length, and the fresh weight of the seedlings. Six replicates were conducted for each treatment, with 15 chili pepper seeds per replication.

2.7. Statistical analysis

The data underwent one-way analysis of variance (ANOVA), and Tukey's HSD test was employed to ascertain statistically significant differences between treated samples and the untreated control, with a significance threshold set at $p < 0.05$.

3. Results

3.1. In vitro evaluation of the mycelial growth of strain PSU-03 using both strains SKRU-01 and NST-009

Building upon the biological facts outlined above, *in vitro* experiments were conducted to enhance our understanding of the competitive and biocontrol capabilities of strains SKRU-01 and NST-009 against PSU-03 (Fig. 1). In the *in vitro* dual culture on Petri plates, both strains SKRU-01 (Fig. 1B) and NST-009 (Fig. 1D) effectively arrested the mycelial growth of PSU-03, eventually completely covering the PSU-03 colony. In comparison to PSU-03, SKRU-01 (Fig. 1A) and NST-009 (Fig. 1C) colonized a greater area of the culture medium as a result of their more rapid mycelial growth. Prior to physical contact between hyphae, both strains SKRU-01 and NST-009 exhibit a narrow region of mycelial growth inhibition against PSU-03. This inhibition is substantial, with maximum mycelial inhibition percentages of 63.21% and 66.60%, respectively, in comparison to the control treatment. The plate confrontation assay results indicated that both strains SKRU-01 (Fig. 1B) and NST-009 (Fig. 1D) exhibited overgrowth and spore production on PSU-03 10 weeks after inoculation at 30 °C.

A comparison of the antifungal activity of two culture filtrates, SKRU-01 and NST-009, with five chemical fungicides (prochloraz®, metalaxyl®, azoxystrobin®, propiconazole®, and thiram®), with respect to their ability to inhibit mycelial growth in PSU-03, is illustrated in Table 1 and Fig. 2. The findings of the study revealed that culture filtrates SKRU-01 (80.72%) inhibited the growth of PSU-03 significantly more effectively than metalaxyl® (76.81%), azoxystrobin® (74.40%), and thiram® (70.48%) ($p < 0.05$). However, their activity was weaker than that of culture filtrates NST-009, propiconazole®, and prochloraz®, which exhibited complete inhibition (100%) of PSU-03. Moreover, both culture filtrates SKRU-01 and NST-009 demonstrated a robust inhibitory effect on the mycelial growth of PSU-03. The inhibitory effect exceeded 50% even when the culture filtrate was diluted at 1/

Table 1
The impact of culture filtrates SKRU-01, culture filtrates NST-009, and chemical fungicides (prochloraz®, metalaxyl® azoxystrobin®, propiconazole®, and thiram®) on the mycelial growth of *Colletotrichum gloeosporioides* PSU-03 in PDA medium after seven days incubation at 30 °C.

Treatments	Radial growth (cm)	Inhibition (%)
Control	5.53^{a}±0.26	–
Undiluted culture filtrates SKRU-01	1.07^{h} ± 0.15	80.72
1/10 diluted culture filtrates SKRU-01	1.78^{de} ± 0.17	67.77
1/100 diluted culture filtrates SKRU-01	2.10^{cd} ± 0.28	62.05
1/1000 diluted culture filtrates SKRU-01	2.15^{c}±0.14	61.14
Autoclaved culture filtrates SKRU-01	2.32^{bc}±0.18	58.13
Undiluted culture filtrates NST-0096	0.00^{i}±0.00	100.00
1/10 diluted culture filtrates NST-009	2.13^{c}±0.23	61.45
1/100 diluted culture filtrates NST-009	2.42^{bc}±0.16	56.33
1/1000 diluted culture filtrates NST-009	2.52^{b} ± 0.04	54.52
Autoclaved culture filtrates NST-009	2.52^{b} ± 0.04	54.52
Propiconazole® (25% (w/v))	0.00^{i}±0.00	100.00
Prochloraz® (45% (w/v))	0.00^{i}±0.00	100.00
Metalaxyl® (25% (wp))	1.28^{gh} ± 0.20	76.81
Azoxystrobin® (25% (w/v))	1.42^{fg} ± 0.23	74.40
Thiram® (80% (wg))	1.63^{ef}±0.15	70.48

Note: Reported values represent means ± standard deviation (SD) from three replicates. Data followed by the same letter within each column show no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$).

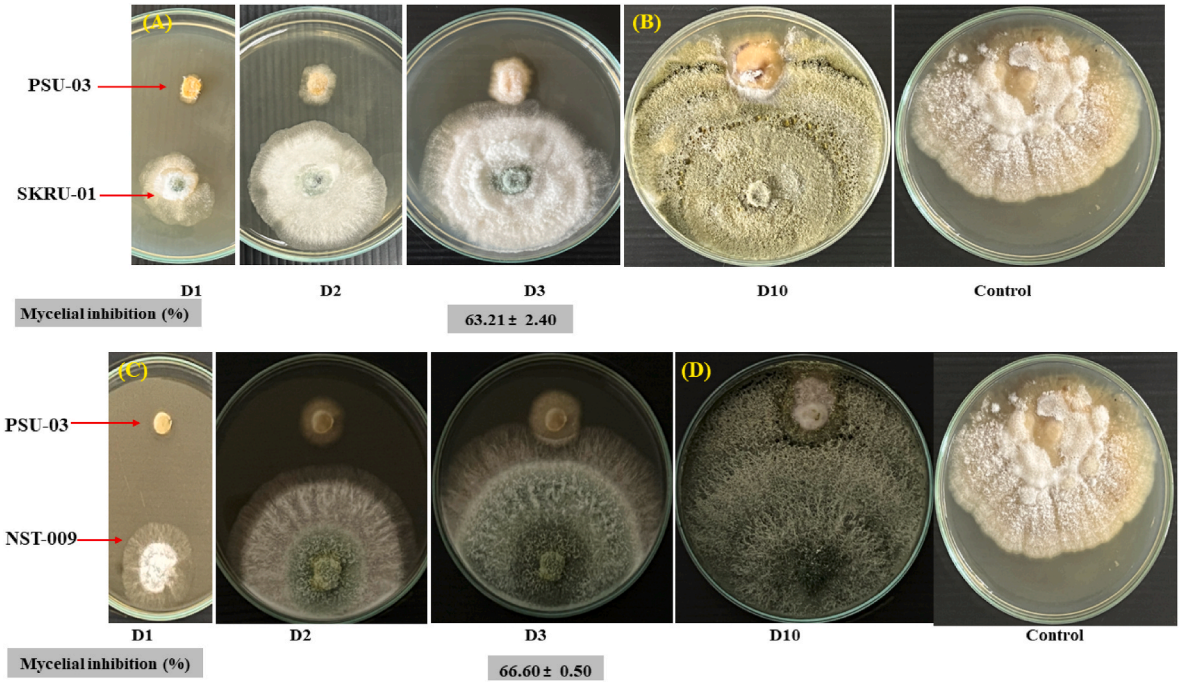

Fig. 1. Development of the mycoparasitic reaction of *Trichoderma asperelloides* SKRU-01 and *T. asperellum* NST-009 against *Colletotrichum gloeosporioides* PSU-03. Growth kinetic of strain SKRU-01 and strain PSU-03 **(A)** and strain NST-09 and strain PSU-03 **(C)** on PDA medium for 3 days. Plate confrontation assays where both strain SKRU-01 **(B)** and NST-09 **(D)** mycelial overgrew and sporulated on strain PSU-03 ten days after inoculation 30 °C.

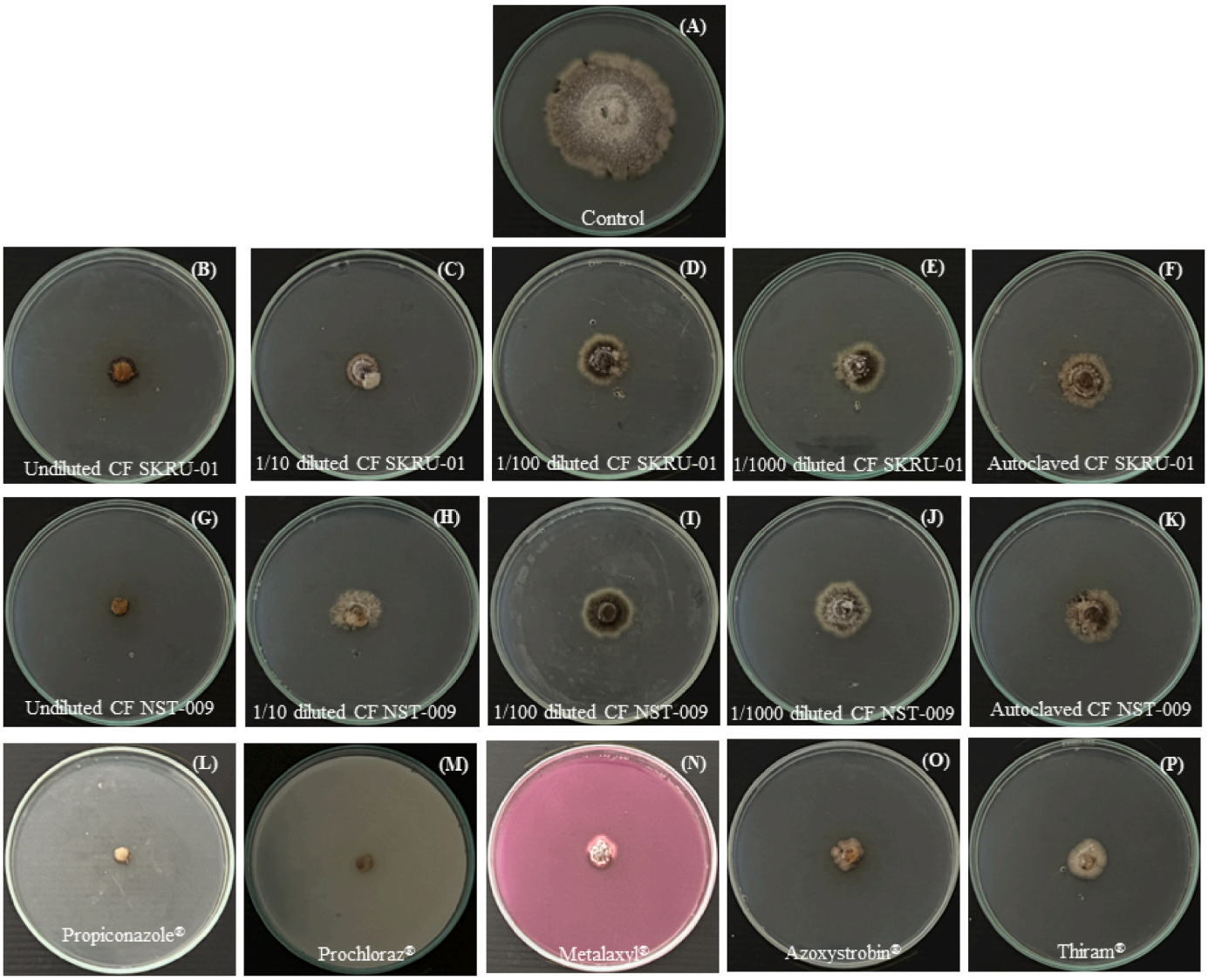

Fig. 2. Comparison of the antifungal activity of both culture filtrates (CF) SKRU-01 and NST-009 with five chemical fungicides (prochloraz®, metalaxyl®, azoxystrobin®, propiconazole®, and thiram®) on the mycelial growth of *Colletotrichum gloeosporioides* PSU-03 in PDA medium after seven days of incubation at 30 °C. **(A)** Control; **(B)** Undiluted culture filtrates SKRU-01; **(C)** 1/10 diluted culture filtrates SKRU-01; **(D)** 1/100 diluted culture filtrates SKRU-01; **(E)** 1/1000 diluted culture filtrates SKRU-01; **(F)** Autoclaved culture filtrates SKRU-01; **(G)** Undiluted culture filtrates NST-0096; **(H)** 1/10 diluted culture filtrates NST-009; **(I)** 1/100 diluted culture filtrates NST-009; **(J)** 1/1000 diluted culture filtrates NST-009; **(K)** Autoclaved culture filtrates NST-009; **(L)** Propiconazole®; **(M)** Prochloraz®; **(N)** Metalaxyl®; **(O)** Azoxystrobin®; **(P)** Thiram®.

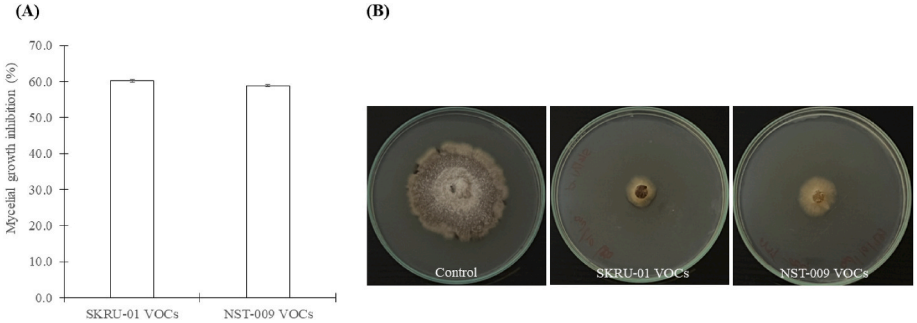

Fig. 3. Inhibition of **(A)** the mycelial growth of *Colletotrichum gloeosporioides* PSU-03 by volatile organic compounds (VOCs) produced by *Trichoderma asperelloides* SKRU-01 and *T. asperellum* NST-009 (SKRU-01 VOCs or NST-009 VOCs) after inoculation at 30 °C for seven days. **(B)** shows the colony morphology of strain PSU-03 after treatment with SKRU-01 VOCs or NST-009 VOCs.

1000 or autoclaved at 121 °C for 15 min, indicating a potent antifungal effect of the two strains in both culture filtrates SKRU-01 and NST-009 tested. The colony morphology of PSU-03 after treatment with both culture filtrates SKRU-01 and NST-009, as well as five chemical fungicides, is illustrated in Fig. 2.

Fumigation with SKRU-01 VOCs and NST-009 VOCs influenced the mycelial growth of PSU-03 (Fig. 3). SKRU-01 VOCs demonstrated a stronger inhibition (60.24%) compared to NST-009 VOCs (58.89%) (Fig. 3A). The colony morphology of PSU-03 after treatment with SKRU-01 VOCs and NST-009 VOCs is depicted in Fig. 1B. The results above suggest that both strains SKRU-01 and NST-009 inhibited strain PSU-03 through competition for space and nutrients, as well as the production of volatile and non-volatile organic inhibitory compounds.

3.2. Biocontrol potential of both strains SKRU-01 and NST-009 against strain PSU-03 on chili fruits

Fig. 4 illustrates the efficacy of biological and chemical controls against strain PSU-03 on chili fruits, comparing the performance of two antagonistic microorganisms, SKRU-01 and NST-009, with that of five chemical fungicides (prochloraz®, metalaxyl®, azoxystrobin®, propiconazole®, and thiram®). Biological and chemical controls significantly ($p < 0.05$) influenced protection against anthracnose disease in chili fruits. Notably, biocontrol treatments with strains SKRU-01 (43.53–60.34% protection) and NST-009 (39.66–62.07% protection) demonstrated superior efficacy in guarding against anthracnose disease compared to chemical fungicides, including metalaxyl® (46.59% protection), azoxystrobin® (45.97% protection), and thiram® (30.34% protection). Interestingly, at a concentration of 1×10^5 spores/mL, strains SKRU-01 (60.34% protection) and NST-009 (62.07% protection) exhibited heightened protection against anthracnose in chili fruits, although their efficacy was surpassed by propiconazole® and prochloraz®, achieving 100% protection. From the conducted experiments, it became evident that using spore suspensions of both strains SKRU-01 and NST-009 was more effective than utilizing the culture filtrates. Consequently, further studies were undertaken using fungal spore suspensions.

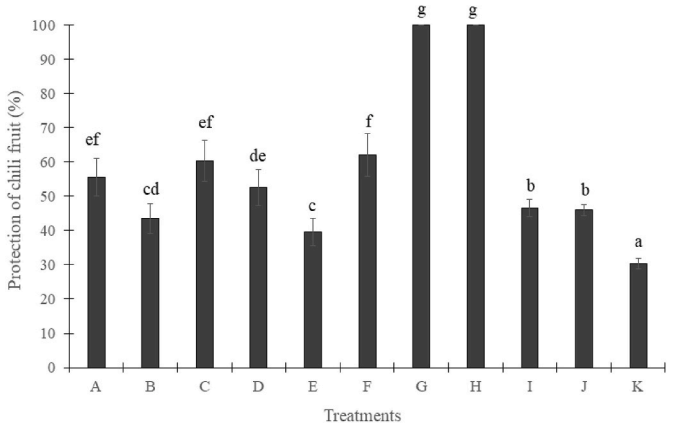

Fig. 4. Percentage protection of chili anthracnose caused by *Colletotrichum gloeosporioides* PSU-03 with the *Trichoderma asperelloides* SKRU-01 and *T. asperellum* NST-009 and five chemical fungicides (prochloraz®, metalaxyl®, azoxystrobin®, propiconazole®, and thiram®) after 10 days of incubation. **(A)** Culture filtrates SKRU-01; **(B)** Autoclaved cultures SKRU-01; **(C)** SKRU-01 spore concentration of 1×10^5 spores/mL; **(D)** Culture filtrates NST-009; **(E)** Autoclaved culture filtrates NST-009; **(F)** NST-009 spore concentration of 1×10^5 spores/mL; **(G)** Propiconazole®; **(H)** Prochloraz®; **(I)** Metalaxyl®, **(J)** Azoxystrobin®; **(K)** Thiram®. The control group is treated by applying sterile distilled water. Values represent the mean (±SD) of three replicates (8 fruits per replicate). Values with the same letter show no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$).

3.3. Impact of varying SKRU-01 and NST-009 spore concentrations against PSU-03 strain on chili fruits

The impact of different spore concentrations (10^5 to 10^8 spores/mL) of both strains, SKRU-01 and NST-009, on controlling anthracnose caused by strain PSU-03 in chili fruits is presented in Fig. 5. Regardless of the spore concentration (10^5 to 10^8 spores/mL) of each strain, RM-1-138 and NST-009 applied to control the PSU-03 strain in chili fruits, none of the spore concentrations exhibited significant protection of chili fruits ($p > 0.05$). Strain SKRU-01 demonstrated a protection range of 57.99%–65.75%, while strain NST-009 displayed a protection range of 59.82%–67.58% against anthracnose disease in chili fruits.

3.4. Preventive and curative efficacy of both strains SKRU-01 and NST-009 against PSU-03 strain on chili fruits

The findings depicted in Fig. 6 showcase the outcomes following the co-inoculation of strain PSU-03 with both strains SKRU-01 and NST-009 into chili fruits. Significantly ($p < 0.05$), the efficacy in anthracnose disease protection in chili fruits hinges on the timing of application for both strains SKRU-01 and NST-009. The application of spore suspension from bioagents specifically strains SKRU-01 and NST-009, on chili fruit wounds concurrently with the inoculation of strain PSU-03 manifests a robust protection index exceeding 66.0%. However, when chili fruits underwent treatment with bioagents SKRU-01 and NST-009 a few hours before PSU-03 inoculation, a significant decrease in protective efficacy was observed ($p < 0.05$). Notably, a treatment administered on wounds with bioagents SKRU-01 and NST-009 6 h before PSU-03 inoculation appears to favor the development of strain PSU-03, resulting in protection indices of 26.03% and 11.87% for strains SKRU-01 and NST-009, respectively. When bioagents SKRU-01 and NST-009 were administered to chili fruits a few hours after inoculation with strain PSU-03, a noteworthy decrease in protective efficacy was observed ($p < 0.05$). The resulting protection indices were less than 8.50% and 10.0% for bioagents SKRU-01 and NST-009, respectively, when inoculated 6 h after the introduction of the fungal pathogen strain PSU-03.

3.5. The effects of culture filtrates SKRU-01 and NST-009 on chili seed germination

Compared to sterile distilled water (control), the effects of culture filtrates SKRU-01 and NST-009 on chili pepper seed germination, stem length, root length, and fresh weight are presented in Table 2 and Fig. 7. Treatment with SKRU-01 significantly ($p < 0.05$) inhibited seed germination (57.78%), stem length (8.20 mm), root length (3.79 mm), and fresh weight (32.56 mg/seedling) compared to the control (93.33% germination, 31.1 mm stem length, 55.5 mm root length, and 59.40 mg/seedling fresh weight). Conversely, culture filtrates NST-009 did not inhibit seed germination (91.11%) and, instead, promoted greater stem length (34.2 mm), root length (64.30 mm), and fresh weight (66.70 mg/seedling).

4. Discussion

Trichoderma species are well-established as biocontrol agents against fungal plant pathogens and have gained recognition as a valuable source of bioactive metabolites. In this study, both strains, *Trichoderma asperelloides* SKRU-01 and *T. asperellum* NST-009, exhibited remarkable and rapid growth, significantly impacting the hyphal growth of *Colletotrichum gloeosporioides* PSU-03 on PDA agar plates. This suggests a robust competition for space and nutrients, resulting in the inhibition and/or parasitization of the pathogen, highlighting their potent antifungal effects. Furthermore, the inhibition of mycelial growth of strain PSU-03 was achieved by the culture filtrates of both strains SKRU-01 and NST-009.

Based on the results obtained from our *in vitro* experiment, it can be

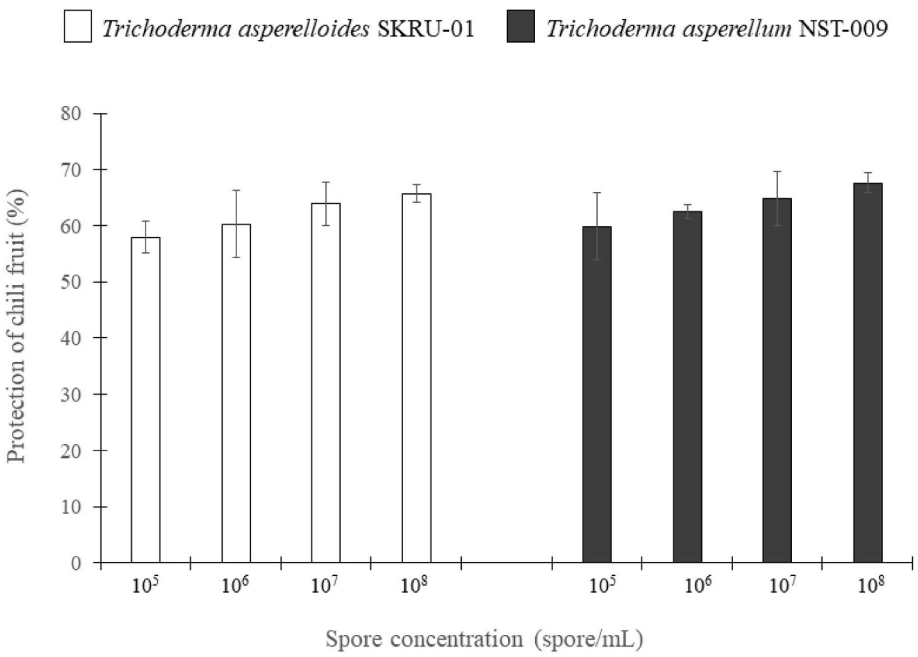

Fig. 5. Percentage protection of chili anthracnose caused by *Colletotrichum gloeosporioides* PSU-03 with spore concentrations of each *Trichoderma asperelloides* SKRU-01 and *T. asperellum* NST-009 (from 10^5 to 10^8 spores/mL) after 10 days of incubation. Values represent the mean (±SD) of three replicates (8 fruits per replicate). Values with the same letter show no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$).

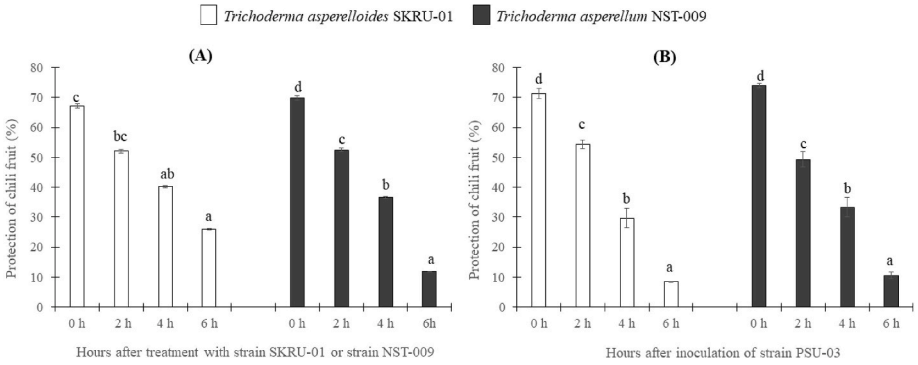

Fig. 6. Timing of preventive **(A)** or curative **(B)** measures by each *Trichoderma asperelloides* SKRU-01 and *T. asperellum* NST-009 on the protection of chili anthracnose caused by *Colletotrichum gloeosporioides* PSU-03 after 10 days of incubation. Values represent the mean (±SD) of three replicates (8 fruits per replicate). Values with the same letter show no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$).

inferred that the culture filtrates of both strains might comprise hazardous secondary metabolites that impede the development of strain PSU-03. Significantly superior to metalaxyl®, azoxystrobin®, and thiram®, these metabolites inhibited the colony growth and spore germination of strain PSU-03. However, they were comparatively less effective than propiconazole® and prochloraz®. The results of our study are consistent with those reported by Abdelmoteleb et al. (2023), which indicate that the mycelial growth of *Fusarium solani* was substantially hindered by culture filtrates derived from *T. longibrachiatum* AD-1. Moreover, culture filtrates of *T. longibrachiatum* also exhibited inhibitory effects on the development of *Magnaporthe oryzae*, *Botrytis cinerea*, and *Phytophthora infestans* (Ngo et al., 2021). The findings presented here are consistent with the research conducted by Yassin et al. (2022), which demonstrate the effectiveness of culture filtrates obtained from *T. viride* and *T. harzianum* against *Alternaria alternata* and *F. proliferatum*, respectively, in inhibiting fungal growth. *Trichoderma* species are acknowledged for their synthesis of a diverse range of bioactive agents, including trichodermin, trichodermil, fitotripen, and trichozam. These compounds demonstrate the ability to inhibit the growth of a broad spectrum of pathogens (Hyder et al., 2017; Rajani et al., 2021).

In the realm of plant disease biological control, biological control agents like *Trichoderma* may not always engage in direct interactions with pathogens or plants. However, volatile compounds (VOCs) play a crucial role in indirect interactions between a biological control agent and plant pathogenic fungi or between the biological control agent and the plant itself. *Trichoderma asperelloides* SKRU-01 has previously showcased its potential in the biological control of food spoilage fungi, particularly those caused by aflatoxigenic *Aspergillus* species in peanuts, by emitting VOCs (Boukaew, Petlamul, Yossan, et al., 2024). This study affirms the broad antifungal VOCs effect of strain SKRU-01, suggesting its wide-ranging potential applications in biocontrol. In VOC assays, both strains SKRU-01 and NST-009 inhibited the growth of strain PSU-03 by 58.89–60.24%, a potentially VOC-driven effect. This level of inhibition surpassed that of other *Trichoderma* species (*T. longibrachiatum*, *T. harzianum*, and *T. pleuroti*), which inhibited *Sclerotinia sclerotiorum* (TSS), *Sclerotium rolfsii* (CSR), and *Fusarium oxysporum* (CFO) to a lesser extent (45–50%). *Trichoderma koningiopsis* T-51, for example, exhibited inhibitory activity against plant pathogenic

Table 2
Percent seed germination, stem length, root length, and fresh weight of chili pepper seeds were measured after soaking them in sterile distilled water, culture filtrates SKRU-01, and culture filtrates NST-009 and incubated in a moist plastic box for ten days.

Treatments	Percentage of seed germination	Stem length (mm)	Root length (mm)	Fresh weight (mg/seedling)
Sterile distilled water (Control)	$93.33^{a}\pm7.30$	$31.1^{a}\pm3.14$	$55.5^{b}\pm8.89$	$59.40^{a}\pm2.46$
Culture filtrates SKRU-01	$57.78^{b}\pm8.07$	$8.20^{b}\pm1.67$	$3.79^{c}\pm0.78$	$32.56^{b}\pm1.30$
Culture filtrates NST-009	$91.11^{a}\pm12.41$	$34.2^{a}\pm3.76$	$64.30^{a}\pm8.30$	$66.70^{a}\pm6.01$

Note: Reported values represent means ± standard deviation (SD) from six replications (15 chili pepper seeds per replication). Data followed by the same letter within each column show no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$).

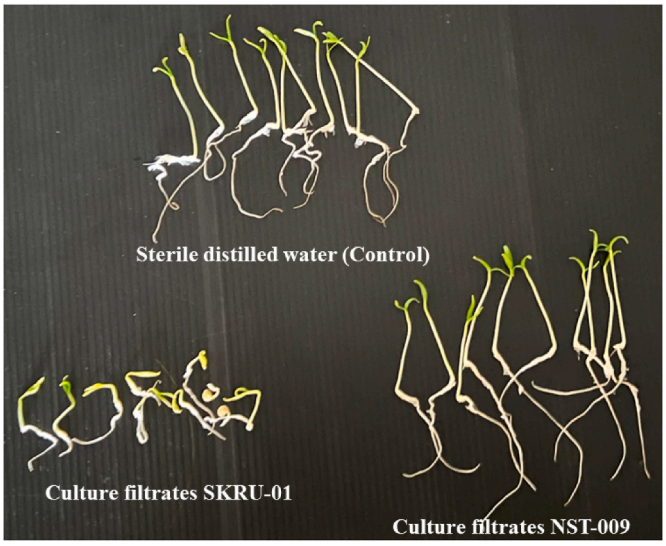

Fig. 7. Illustrates the morphology of chili pepper seeds after soaking in sterile distilled water, culture filtrates SKRU-01, and culture filtrates NST-009, followed by incubation in a moist plastic box for ten days.

fungi *B. cinerea* and *F. oxysporum* (You et al., 2022). Furthermore, Ma et al. (2023) document that *T. asperellum* 576 exhibited the most substantial inhibitory effect on the mycelial growth of *Exserohilum turcicum* 101. The strain SKRU-01 is recognized for producing 23 VOCs, with acetophenone being the predominant compound. These VOCs exert inhibitory effects on *A. parasiticus* and *A. flavus* through various mechanisms, including the inhibition of ergosterol biosynthesis and an impact on antioxidative defense molecules (Boukaew, Petlamul, Yossan, et al., 2024). Calistru et al. (1997) demonstrate that the growth inhibition of *A. flavus* and *Fusarium verticillioides* was attributed to VOCs produced by certain strains of *T. harzianum* and *T. viride*. However, not all *Trichoderma* strains exhibit the ability to inhibit *in vitro* growth through VOC production, as evidenced by the varied responses of different strains in inhibiting the growth of *A. flavus* and *F. moniliforme*.

The utilization of both strains SKRU-01 and NST-009 in combatting chili anthracnose induced by strain PSU-03, in comparison to five chemical fungicides (prochloraz®, metalaxyl®, azoxystrobin®, propiconazole®, and thiram®), demonstrate that biocontrol treatments effectively impeded the progression of anthracnose disease. This inhibition was attributed to the effects of secondary metabolites produced by these fungi. Notably, employing a spore concentration of 1×10^5 spores/mL for each strain, SKRU-01 and NST-009 exhibited superior efficacy in slowing down anthracnose development compared to metalaxyl®, azoxystrobin®, and thiram®, although they were outperformed in effectiveness by propiconazole® and prochloraz®. This suggests that biocontrol treatments, especially when optimized in terms of spore concentration, can serve as a viable alternative or supplement to chemical fungicides in anthracnose disease management. These findings underscore the potential of the spore concentration of both strains SKRU-01 and NST-009 as promising biocontrol agents against anthracnose disease. Consequently, we hypothesized that increasing the spore concentration of both fungal strains would be effective in halting anthracnose development. However, our study revealed that, despite increasing the spore concentration from 10^5 to 10^8 spores/mL, there was no significant difference ($p > 0.05$) in the effectiveness of inhibiting anthracnose disease development in chili fruits. At the tested spore concentrations, both strains SKRU-01 and NST-009 exhibited the strongest protective efficacy against strain PSU-03, with a range of 57.99%–65.75% protection and 59.82%–67.58% protection, respectively. These results suggest that both fungal strains may have alternative potential for use in protecting chili fruits in agricultural fields against postharvest anthracnose disease. The findings of this study confirm that strains belonging to the *Trichoderma* genus have the capacity to regulate chili anthracnose effectively (Ruangwong et al., 2021; Kim et al., 2023; Nujthet et al., 2023). The severity of anthracnose in chili fruits was effectively controlled by both strains SKRU-01 and NST-009 through curative and preventive methods. The optimal protection against strain PSU-03 was achieved when spore suspensions of SKRU-01 and NST-009 were concomitantly inoculated with PSU-03 spores, resulting in a protection index exceeding 67%. However, treating chili fruits with either or both fungal strains before or after PSU-03 inoculation led to a significant ($p < 0.05$) decrease in protective efficacy. The lack of a preventive effect observed in chili fruits against both strains implies that they are incapable of eliciting significant systemic resistance. This may indicate that the strains are susceptible to plant-produced molecules, rendering them unable to persist within the fruits. Additional experiments need to be undertaken to evaluate the strains' capacity to endure in chili tissues and to verify these hypotheses. The absence of a curative effect indicates that the fungi's production of secondary metabolites may be inadequate to impede the growth of strain PSU-03 on chili fruits. This provides support for the hypothesis that the fungus is incapable of thriving on chili fruits. Consequently, further investigation is imperative in order to develop approaches for the preventative or curative utilization of this strain. Consistent with this, Kim et al. (2023) report that red pepper anthracnose caused by *C. acutatum* could be prevented and treated with spores of *T. atroviride* ATR697 and *T. longibraciatum* LON701. Literature indicates that suspensions of *Trichoderma* spores may be utilized to manage a wide range of plant diseases (Dawidziuk et al., 2016; Kim et al., 2023; Sunpapao et al., 2018). Another crucial mechanism of *Trichoderma* species is biostimulation, which induces plant resistance mechanisms and promotes root development. We examined the potential of culture filtrates from both strains SKRU-01 and NST-009 to biostimulate the germination and growth of chili pepper seeds. The culture filtrate derived from SKRU-01 exhibited a notable inhibitory effect on the germination of chili pepper seeds. This effect may be ascribed to the significant concentration of bioactive metabolites present in the filtrates. This study has the same findings as Boukaew et al. (2011), who report a significant inhibitory effect of the culture filtrates of *S. philanthi* RM-1-138 on the germination of seeds and proliferation of seedlings in chili pepper. In contrast, NST-009 did not show any effect on chili pepper seed germination and appeared to promote seed germination. Our findings differ from the results presented by Kim et al. (2023), who assert that *T. asperellum* 576, at a concentration of 10^7 spores/mL, improved maize seed germination. Furthermore, Singh et al. (2016) observe discrepancies in the spore dosage needs of

T. asperellum BHUT8 in order to promote seed germination and radicle length, both of which are crucial for the initial development of diverse vegetable crops. Additional research is required to attain a comprehensive understanding of this subject. Moreover, in addition to bioactive compounds inhibiting plant roots, volatile compounds from *T. harzianum* tri5 have been found to reduce both root and aerial growth of wheat plantlets as well (Álvarez-García et al., 2022).

5. Conclusion

The examination of *Trichoderma asperelloides* SKRU-01 and *T. asperellum* NST-009 in this study reveals their significant potential for postharvest control of chili anthracnose caused by *Colletotrichum gloeosporioides*, both *in vitro* and on chili fruits. These fungi exhibit efficacy via diverse mechanisms, encompassing competition for nutrients and space, in addition to generating compounds that are both volatile and non-volatile in nature. Notably, they exhibit superior efficiency in both protective and curative effects against chili anthracnose. Moreover, the study indicates that culture filtrates SKRU-01 strongly inhibit chili pepper seed germination and seedling growth. While both SKRU-01 and NST-009 hold promise for managing postharvest anthracnose in chili fruits, caution is advised when considering the use of culture filtrates SKRU-01 for biostimulating root development in chili peppers.

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Availability of data and materials

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Sawai Boukaew: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Krittin Chumkaew:** Data curation, Formal analysis, Writing – review & editing. **Wanida Petlamul:** Writing – review & editing, Funding acquisition, Formal analysis, Data curation. **Sirasit Srinuanpan:** Writing – review & editing. **Karistsapol Nooprom:** Writing – review & editing, Formal analysis. **Zhiwei Zhang:** Writing – review & editing, Formal analysis.

Declaration of competing interest

The authors declare no competing interests.

Data availability

The authors do not have permission to share data.

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