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Metabolic Adaptation of Trichoderma Asperellum Wild-Type to Enhance Growth Rate and Biocontrol Efficacy against Anthracnose (Colletotrichum spp.)

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Abstract:- Pathogenic fungi significantly damage crops in both vegetable and fruit cultivation. Trichoderma asperellum is a biocontrol fungus that suppresses these pathogens and promotes plant growth. However, its effectiveness in controlling such pathogens is limited. This study aimed to enhance the biocontrol efficacy of T. asperellum against crop-damaging fungi through metabolic adaptation. Serial transfer experiments were conducted in potato dextrose broth (PD broth) containing 3% sugar, followed by plating with anthracnose to compare the inhibitory effects of the wild-type strain and its evolved variant. The evolved strain, T. asperellum TIS-11T, demonstrated significantly improved growth and sugar utilization, reaching a biomass of 0.5 g/mL within 48 hours. Additionally, the evolved strain exhibited complete inhibition (100%) of anthracnose on PDA culture plates within 5 days. One-way ANOVA revealed a significant difference ($p<0.01$) increase in growth inhibition by the T. asperellum TIS-11T strain compared to the wild-type. In conclusion, T. asperellum TIS-11T is a highly effective biocontrol agent against anthracnose and has strong potential for industrial applications in pathogen management.

Keywords:- *Metabolic Adaptation, Trichoderma Asperellum, Growth Rate, Biocontrol, Anthracnose.*

I. INTRODUCTION

Cambodia is an agricultural country, with about 61% of its population living in rural areas and approximately 77% relying on agriculture, including activities such as farming, fishing, and animal husbandry (Karamba et al. 2022). Cambodian agriculture is heavily impacted by climate change, which brings increased temperatures, irregular rainfall, and rapid weather changes (Karamba et al. 2022). Additionally, various crops produced in Cambodia, including fruits and vegetables like bananas, chilies, mangoes, and others, are exported abroad (Piseth et al. 2021). Despite this, all crops are vulnerable to damage during the production cycle, including planting, care, and harvest. Factors contributing to reduced crop yields and losses include agricultural practices, biological factors, and environmental conditions (Liliane et al. 2020). Among the most destructive factors affecting crops is fungal pathogens that cause anthracnose, a disease that frequently occurs and destroys crops annually, whether they are leafy plants or fruit-bearing plants, despite the use of pesticides during planting and after harvest (Alves et al. 2015 & Peralta-Ruiz et al. 2023). In tropical regions, anthracnose causes fruit rot and is commonly found on crops like pepper, tomatoes, and many other agricultural products (Zakaria 2021). To control and treat this issue, farmers have been using chemical pesticides, but the use of these chemicals has been found to have negative effects on the crops, especially by interfering with the photosynthesis process, causing a decline in the crops' growth and yield (Petit et al. 2012). Chemical pesticides also contribute to pollution, the evolution of resistant pathogens, and serious health risks to humans and other living organisms (Goswami et al. 2018). Thus, controlling biological factors with the use of biologically derived pesticides has become an important requirement for agricultural productivity (dos Santos et al. 2021). Recently, biological pesticides have played a key role in replacing chemical pesticides, offering effectiveness without impacting human health, animals, or crops. Biological pesticides contain beneficial organisms, including fungi and bacteria, that combat harmful fungal and bacterial diseases. Among these, Trichoderma species are popular and effective in controlling fungal and bacterial pathogens that cause diseases like rot, leaf blight, and fruit spoilage. Moreover, Trichoderma not only protects and treats diseases but also promotes plant growth. Biological pesticides produced from *Trichoderma* are safe, cost-effective, and efficient for crops (Yao et al. 2023). Despite this, the effectiveness of Trichoderma remains limited for practical use, leading farmers to continue using chemical pesticides for pathogen control (Supyani et al. 2023). Similarly, Ons et al. (2020) pointed out that over 78,000 farmers worldwide require agricultural pesticides for fungal disease treatment and still use chemical pesticides more frequently due to their higher effectiveness. Ruangwong et al. (2021a) found that Trichoderma produces organic compounds that inhibit the growth of pathogenic fungi, meaning that fungal pathogens are weakened by substances

produced by Trichoderma. Similarly, metabolic evolution of beneficial microorganisms is a popular method to enhance microbial growth and performance (In et al. 2020). The culture of Trichoderma under both biotic and abiotic stress conditions leads to improved strains with higher capabilities, contributing positively to crop production (Cabral-Miramontes et al. 2022).

Thus, the purpose of this study is to enhance the growth and inhibitory activity of Trichoderma by cultivating it in high-sugar (3%) potato dextrose broth (PDA) and investigating its effectiveness in controlling anthracnose disease on crops.

II. MATERIALS AND METHODS

A. Potato Dextrose Broth

Potatoes were cleaned, sliced, and 200 grams were boiled in water for 10 minutes. The volume was then adjusted to 1000 mL with water, and sucrose was added at concentrations of 2%, 2.5%, and 3%. The mixture was stirred thoroughly to ensure complete dissolution. The solution was transferred to a glass container and autoclaved at 121°C and 106 kPa (1 atm) for 20 minutes. After sterilization, the broth was cooled and made ready for experimental use.

B. Metabolic Adaptation

The *T. asperellum* wt was obtained from the Microbiology Laboratory at the University of Kratie. Using a serial transfer method, the *T. asperellum* strain was inoculated into the prepared broth and monitored daily until all sucrose was depleted. Cultures were grown in 250 mL flasks containing 100 mL of broth, maintained at 37°C in a shaking incubator set to 200 rpm. The initial biomass concentration was 0.01 g/mL, following the protocol described by In et al. (2020).

C. Percentage Inhibition Measurement

The *T. asperellum* wt and *T. asperellum* TIS-11T, which were enhanced for their biocontrol capabilities, were cultivated on growth media containing the anthracnose pathogenic fungus (*Colletotrichum* spp.). The growth rate of the anthracnose pathogenic fungus was measured every 24 hours until the pathogen was completely suppressed. To determine the percentage of growth inhibition, the formula I = (C-T)/C x 100 was used, where I represents the percentage of inhibition (%), C is the radial growth of *Colletotrichum* spp. (in millimeters), and T is the radial growth of *T. asperellum* (in millimeters) (Rahman et al. 2009).

➢ *The Experiment was Divided into three Treatments:*

- Treatment 1 (TC): Potato dextrose agar (PDA) plates containing *Colletotrichum* spp.
- Treatment 2 (TB): PDA plates containing *Colletotrichum* spp. and *T. asperellum* wt.
- Treatment 3 (TA): PDA plates containing *Colletotrichum* spp. and *T. asperellum* TIS-11T

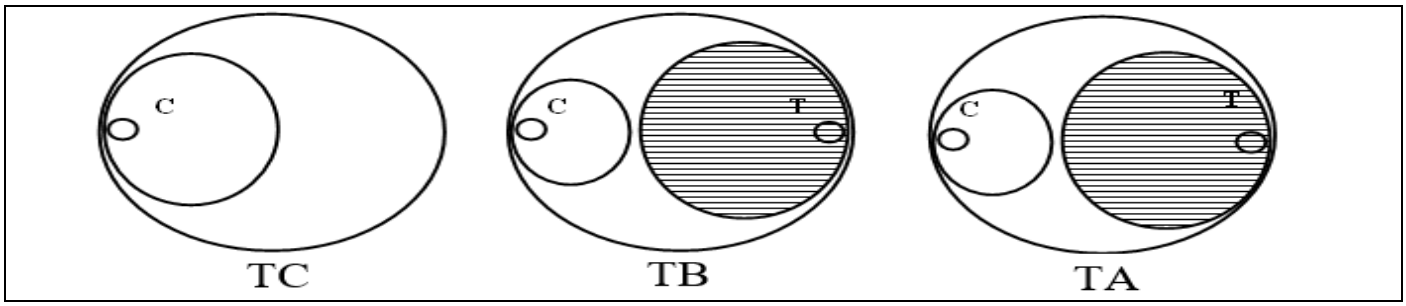

Fig 1: Growth Plates of the Three Factors to Illustrate the Level of Activity Inhibition by the Biocontrol Agents

D. Data Collection

Primary data were collected and analyzed at each experimental stage to obtain numerical and quantitative measurements. The biomass of *T. asperellum* wt was measured every 24 hours until the sugar in the medium was completely depleted. For each measurement, a 1 mL liquid sample was taken and centrifuged using a Mini Centrifuge (Model: Mini-6KS) for 3 minutes. The supernatant was discarded, and the remaining solid residue was weighed using an electronic balance (Model: HL-300LWP). The inhibition level was determined by measuring the radial growth of the anthracnose pathogenic fungus using a millimeter-scale ruler. Measurements were taken every 24 hours for all treatments and replicates. The inhibition percentage for each factor and treatment was calculated using the formula described by Rahman et al. (2009).

E. Data Analysis

All data were entered into Microsoft Excel Professional Plus 2021 for cleaning and proper organization. The data were presented as mean ± standard deviation (SD). The means of the treatments were analyzed using a One-way ANOVA with Tukey's HSD multiple comparisons test. This analysis was performed using GraphPad Prism software (Version 10.2.0, Windows, San Diego, California, USA, www.graphpad.com), with a significance level set at $p < 0.01$.

III. RESULT AND DISCUSSION

A. Metabolic Adaptation of T. Asperellum wt to T. Asperellum TIS-11T

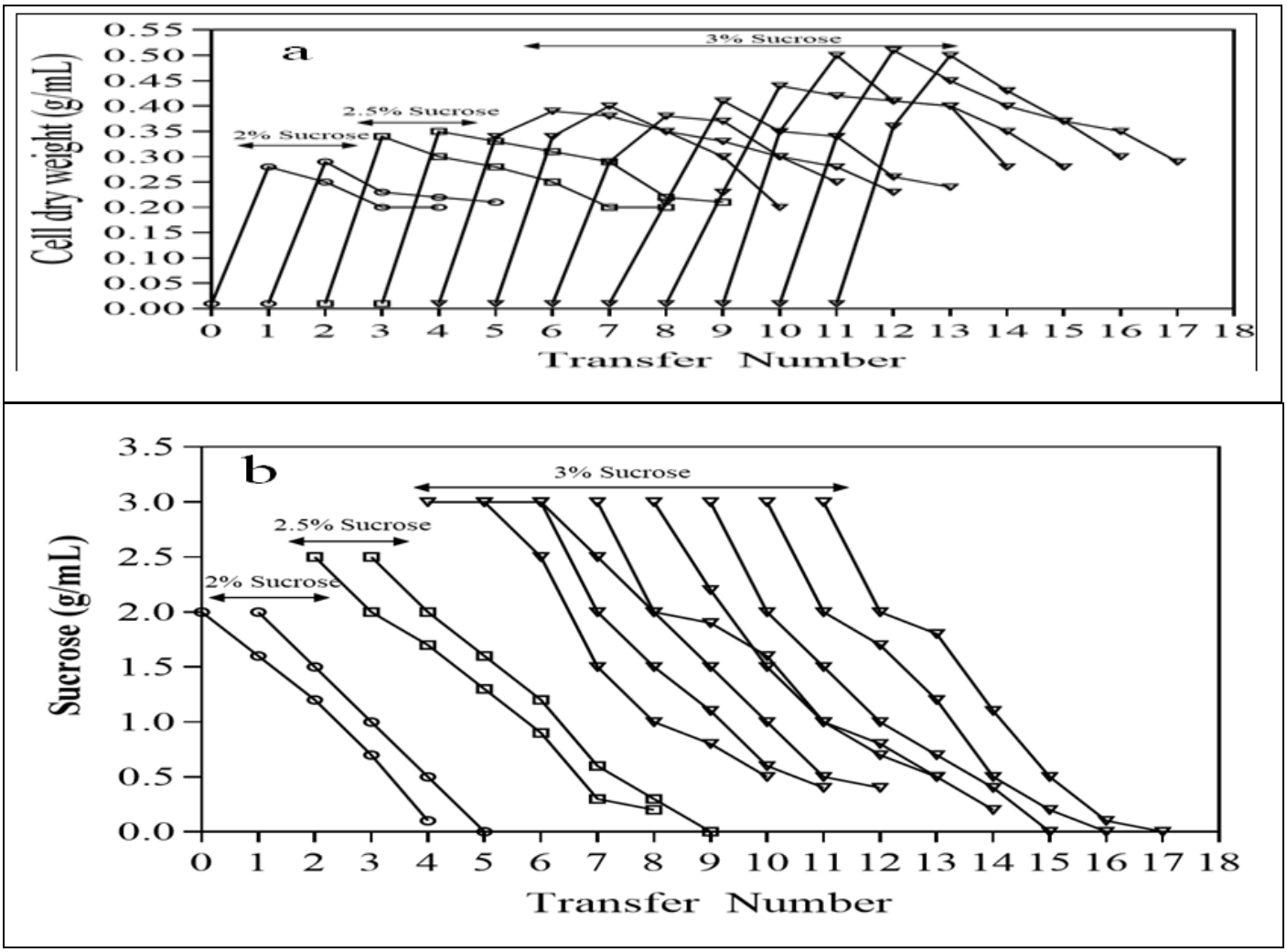

Fig 2: Metabolic Adaptation of T. Asperellum wt in Liquid Culture Media with Sucrose Concentrations Ranging from 2% to 3% (w/v). The Transfers were Conducted from the First to the 11th, Starting with a Biomass Level of 0.01 g/mL. The First and Second Transfers used 2% Sucrose (Circle), the Third and Fourth Transfers used 2.5% Sucrose (Square), and Transfers 4 Through 11 used 3% Sucrose (Triangle). (a) Graph of Biomass (g/mL) and (b) Graph of Percentage sucrose Consumption during Successive Transfers.

After culturing and isolating *T. asperellum* wt, metabolic adaptation was performed through serial transfers in liquid media containing sucrose concentrations ranging from 2% to 3% (w/v). The strain utilizing 3% sucrose most efficiently and showing optimal growth was selected for further evaluation. *T. asperellum* wt was initially cultured in a medium containing 2 g/mL sucrose, starting with a biomass of 0.01 g/mL. By the 11th transfer, biomass progressively increased while sucrose was fully utilized (***Fig. 2***). To optimize growth, a sucrose concentration of 2.5% was applied, enriching the substrate for metabolic adaptation. By the second transfer, biomass levels increased as sucrose concentration rose; however, sucrose consumption was incomplete. By the third transfer, biomass reached 0.35 g/mL, with complete utilization of 2.5% sucrose. From the fourth transfer onward, growth improvements were observed, culminating in a biomass of 0.5 g/mL and complete sucrose utilization at the 11th transfer (***Fig. 2***). These results align with findings from Jantama et al. (2008), which highlighted that microorganism grown in low-sugar environments exhibit reduced metabolic activity and energy production, limiting cell proliferation. The gradual increase in sucrose concentration (to 3%) in subsequent transfers supported enhanced growth and metabolic efficiency, consistent with other studies. The final strain, *T. asperellum* TIS-11T, demonstrated robust growth and a high tolerance for elevated sucrose concentrations, making it a suitable candidate for biocontrol applications against anthracnose pathogens. Additionally, it exhibited superior metabolic adaptation, enabling complete sucrose utilization and sustained biomass production. High substrate concentrations typically inhibit enzymatic activity in microorganisms, as reported by Ghaly et al. (2005).

However, this study found that *T. asperellum* TIS-11T maintained high metabolic activity and growth under these conditions. These findings are consistent with In et al. (2020), who showed that microorganism adaptation in specific media can induce metabolic shifts that enhance growth. Similarly, stress conditions during culturing may improve environmental tolerance and resilience, leading to increased growth (Carroll & Marx 2013 & Jantama et al. 2015).

In conclusion, the metabolic adaptation process resulted in the development of *T. asperellum* TIS-11T, a strain with enhanced growth potential, high biomass production, and complete sucrose utilization. This strain holds significant promise for biocontrol applications and further studies.

B. Percentage of Inhibition of T. Asperellum TIS-11T and T. Asperellum wt to Colletotrichum spp.

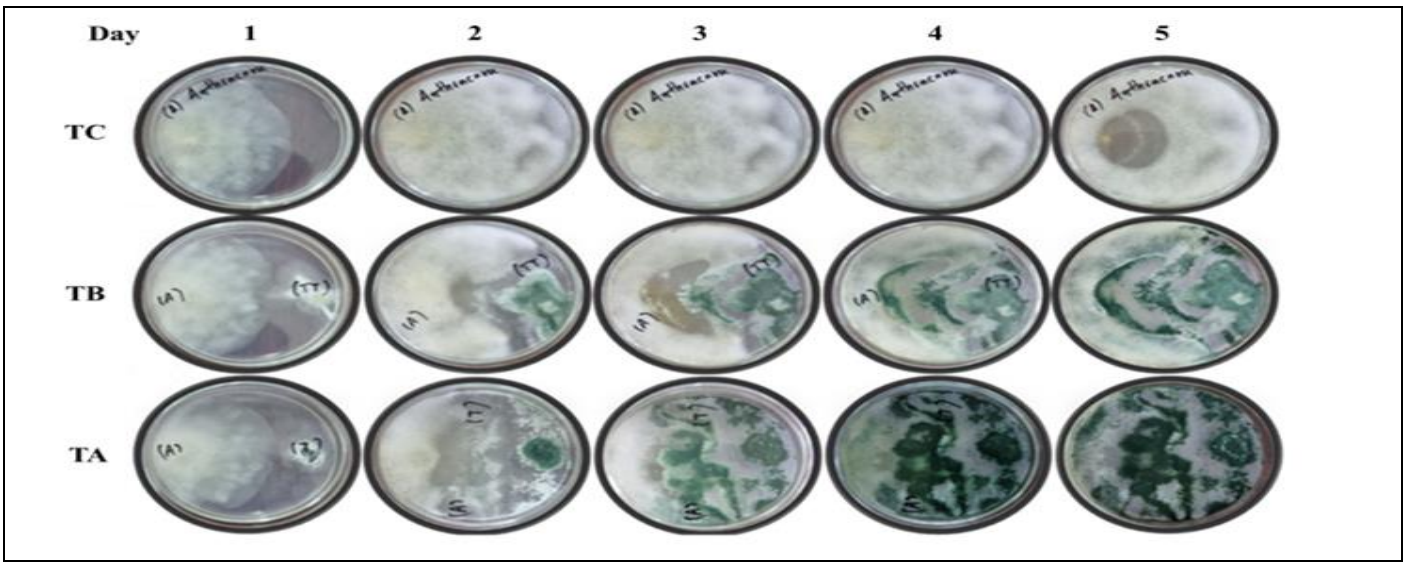

Fig 3: Cultivation of Anthracnose Pathogenic Fungus Colletotrichum spp., T. asperellum wt, and T. asperellum TIS-11T on potato dextrose agar (PDA). TC = Colletotrichum spp. without T. asperellum, TB = Colletotrichum spp. with T. asperellum wt, TA = Colletotrichum spp. with T. asperellum TIS-11T.

The anthracnose pathogenic fungus (Colletotrichum spp.), *T. asperellum* wt, and *T. asperellum* TIS-11T were cultured on solid potato dextrose agar (PDA). The first plate, inoculated with only the anthracnose pathogen, showed strong growth up to the second day with a radial growth of 80 ± 0.01 mm, covering the entire plate (***Fig. 3*** and ***Table 1***). This result is consistent with the findings of Li et al. (2021), who showed that *Colletotrichum* spp. is a fungus that is difficult to control, especially without protection or immediate treatment. Research by López-Moral et al. (2020) confirmed that *Colletotrichum* spp. grows well under sufficient conditions and nutrition, leading to rapid growth without protection.

Table 1: Measurement of Anthracnose Pathogenic Fungus Growth Levels for all Three Treatments.

Day	TC ± SD (mm)	TB ± SD (mm)	TA ± SD (mm)	PI.TB (%)	PI.TA (%)
1	60.30 ± 1.53	54.70 ± 0.58	55.33 ± 0.58	10.38	9.29
2	80.00 ± 0.00	51.30 ± 1.15	29.00 ± 1.00	35.83	63.75
3	80.00 ± 0.00	33.70 ± 3.21	15.33 ± 0.58	57.92	80.83
4	80.00 ± 0.00	16.30 ± 1.53	3.67 ± 1.53	79.58	95.42
5	80.00 ± 0.00	8.00 ± 2.65	0.00	90.00	100.00

TC = Colletotrichum spp. without T. asperellum
TB = Colletotrichum spp. with T. asperellum wt
TA = Colletotrichum spp. with T. asperellum TIS-11T
PI.TB = Percentage of inhibition of TB
PI.TA = Percentage of inhibition of TA
SD = Standard deviation

On the second day, the plate with *T. asperellum* wt showed inhibition of the anthracnose pathogen, with a growth of 51.3 ± 1.15 mm, indicating an inhibition percentage of 35.23%. By the final day, *T. asperellum* wt reduced the radial growth of Colletotrichum spp. to 8 ± 2.65 mm, corresponding to an inhibition percentage of 90%. In the plate with *T. asperellum* TIS-11T, the growth of the anthracnose pathogen was inhibited to 29 ± 1 mm on the second day. On subsequent days, the growth of

Colletotrichum spp. was completely inhibited by *T. asperellum* TIS-11T by the fifth day, with 100% inhibition (***Table 1***).

The growth of the anthracnose pathogen across the three treatments differed significantly ($p<0.01$). The growth of *T. asperellum* wt was optimal at a sucrose concentration of 20 g/L, which led to effective inhibition of anthracnose disease in this experiment (Ajam et al. 2023). A study by Intana et al. (2021) found that *T. asperellum* produces bioactive compounds that are effective against both fungal and bacterial pathogens. This is why the presence of *T. asperellum* slowed down the growth of the anthracnose pathogen and gradually destroyed its mycelial structure. *T. asperellum* TIS-11T exhibited high efficacy in inhibiting the anthracnose pathogen, with complete mycelial destruction in just 5 days following its enhanced metabolic capacity. The increased sucrose concentration of 3% enhanced the growth and metabolism of *T. asperellum* TIS-11T. This finding is in line with the study by In et al. (2020), which showed an increase in biomass following metabolic adaptation. The bioactive compounds produced by *T. asperellum* TIS-11T showed an increase in antifungal activity, contributing to a stronger inhibition of anthracnose. The study by Shang et al. (2020) also demonstrated that *T. asperellum* is highly effective in protecting and treating anthracnose disease. The results of this experiment indicate that *T. asperellum* TIS-11T has improved efficacy in inhibiting anthracnose compared to *T. asperellum* wt, which had not yet shown enhanced capabilities.

C. Comparison of T. asprellum TIS-11T to Recent Studies

Table 2: Comparison of T. asperellum TIS-11T with Other Microorganisms in Inhibiting Colletotrichum spp. Activity.

Strain	Media	Time (Day)	Percentage)%(	References
T. asperellum wt	PDA[a]	5	90	This study
T. asperellum TIS-11T	PDA	5	100	
T. kingingiopsis	PDA	5	79.57	Ruangwong et al.)2021b(
T. harzianum	PDA	7	64.2	Sutarman et al.)2021(
T. asperellum	PDA	7	80	Kim et al.)2023(
T. viride	PDA	10	62.61	Agnihotri et al.)2023(
T. asperellum	PDA	7	76.47	Manzar et al.)2021(
T. harzianum	PDA	5	83	Intana et al.)2007(
Paenibacillus polymyxa AT4	NA[b]	7	76.44	Tram et al. (2023)
Bacillus velezensis AK-0	NA	7	80.70	Kim et al. (2021)
Paenibacillus polymyxa C1	NA	7	100	Suprapta (2022)

[a]Media for fungi (potato dextrose agar)
[b]Media for bacteria (nutrient agar)

Table 2 presents a comparison of the effectiveness of the inhibition of anthracnose disease activity between *T. asperellum* TIS-11T and other recent studies. The level of inhibition was measured based on the percentage of activity inhibition and the duration of the process. Among the biocontrol fungi *Trichoderma* spp. and other bacteria, *T. asperellum* TIS-11T showed the highest effectiveness, with a 100% inhibition level achieved in 5 days. This result aligns with previous studies, which showed that *T. asperellum* wt is a biocontrol agent capable of inhibiting the growth of anthracnose-causing fungi, with a 90% inhibition level in 5 days.

Other *Trichoderma* species such as *T. kingingiopsis*, *T. harzianum*, and *T. viride* showed moderate levels of inhibition. *T. kingingiopsis* exhibited a 79.57% inhibition level in 5 days (Ruangwong et al. 2021), *T. harzianum* showed 83% inhibition in 5 days (Intana et al. 2007), and *T. viride* had a 62.61% inhibition level in 10 days (Agnihotri et al. 2022).

In addition to biocontrol fungi, certain bacteria have also shown high efficacy in inhibiting anthracnose disease activity. However, the level of inhibition and the time required were lower compared to the biocontrol agent *T. asperellum* TIS-11T. Notable bacteria with high efficacy include *Paenibacillus polymyxa* AT4, *Bacillus velezensis AK-0, and Paenibacillus polymyxa C1*. *Paenibacillus polymyxa* AT4 showed a 76.44% inhibition, *Bacillus velezensis* AK-0 had an 80.70% inhibition, and *Paenibacillus polymyxa* C1 showed 100% inhibition in 7 days (Kim et al. 2021; Suprapta et al. 2022; Tram et al. 2023).

When comparing biocontrol agents, both bacteria and *Trichoderma* species, *T. asperellum* TIS-11T in this study demonstrated the highest efficacy and the shortest time for inhibiting anthracnose disease activity.

IV. CONCLUSION

The study on the enhancement of growth capacity through metabolic adaptation of *T. asperellum* TIS-11T was conducted to increase the effectiveness of inhibiting anthracnose disease activity. The results showed that *T. asperellum* wt growth increased from 0.28 g/mL to 0.50 g/mL, and sugar consumption increased from 2% to 3%. Regarding the effectiveness in controlling anthracnose disease, a significant increase was observed, with *T. asperellum* TIS-11T being able to completely eliminate the disease within 5 days. *T. asperellum* TIS-11T demonstrated higher effectiveness compared to recent studies on

anthracnose disease inhibition. We can conclude that *T. asperellum* TIS-11T is a highly potential biocontrol agent for managing anthracnose fungal diseases and promoting sustainable agricultural practices.

This study demonstrates the potential use of *T. asperellum* TIS-11T in laboratory experiments. However, a limitation of this study is that it has not yet been applied directly to crops. In general crop cultivation, it is difficult to control all environmental conditions. Additionally, other types of fungal pathogens can occur on crops, which is a limitation of this research. Therefore, future research should address these limitations by studying the direct application of *T. asperellum* TIS-11T to specific crops and using it to inhibit fungal or bacterial pathogens, thereby reducing the reliance on chemical pesticides.

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