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Antagonistic Evaluation of Trichoderma against The Pathogens Colletotrichum, Pestalotiopsis, and Phytophthora using Dual Culture and Split Plate Techniques

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Abstract

Plant pathogens such as Colletotrichum, Pestalotiopsis, and Phytophthora are major causal agents of significant yield losses in tropical horticultural crops. Among various biological control agents, Trichoderma species have been widely studied due to their antagonistic capabilities, rapid colonization, and ability to produce hydrolytic enzymes. Dual-culture and split-plate assays are commonly used to evaluate direct inhibition mechanisms and inhibition mediated by volatile compounds. This study aimed to evaluate the antagonistic potential of Trichoderma reesei against three tropical pathogens using both assay methods. The antagonistic potential of Trichoderma reesei against three major tropical plant pathogens—Colletotrichum gloeosporioides, Pestalotiopsis microspora, and Phytophthora palmivora—was assessed using dual-culture and split-plate techniques. In the dual-culture assay, T. reesei showed varying levels of inhibition across the tested pathogens. The highest mean inhibition was observed against P. microspora (89.37 ± 1.48%), followed by C. gloeosporioides (87.22 ± 2.01%), and the lowest against P. palmivora (84.37 ± 1.35%). Statistical analysis (GLM, $F = 9.38$; $p = 0.0063$) revealed significant differences among pathogens, with Tukey's test indicating that inhibition against Pestalotiopsis was significantly greater than against Phytophthora palmivora. The results indicate that Trichoderma reesei exhibits more effective antagonistic activity against Pestalotiopsis than against Phytophthora palmivora. This suggests that the inhibitory mechanisms of T. reesei are more specific and potent against certain pathogens, particularly necrotrophic fungi such as Pestalotiopsis. This specificity enhances its value as a biological control agent by demonstrating selectivity toward target pathogens. Another advantage of T. reesei is its ability to produce hydrolytic enzymes, such as chitinase and glucanase, that degrade the cell walls of pathogens. This enzymatic activity strengthens the effectiveness of biological inhibition without causing negative environmental impacts. Therefore, T. reesei has strong potential as an environmentally friendly alternative to synthetic chemical fungicides. Users are encouraged to integrate T. reesei with Integrated Pest Management (IPM) practices. Combining it with proper cultivation techniques—such as field sanitation, crop rotation, and the use of resistant varieties—will enhance overall disease control effectiveness. Additionally, environmental factors such as humidity, temperature, and soil pH should be considered, as they influence the activity of T. reesei. Optimizing these conditions will improve colonization and antagonistic activity of this microorganism in the field.

Keywords: Antagonistic, *Colletotrichum sp, Pestalotiopsis sp, Phytophthora sp, Trichoderma sp*

1. Introduction

Plant disease pathogens are a major limiting factor in crop production. These pathogens can cause various diseases, including leaf spot, leaf rust, stem canker, and wilt, the latter often caused by the fungus Fusarium species (Hanif & Zamriyetti, 2023). They can enter plants through wounds or be transmitted via seeds.

Colletotrichum sp. is a pathogenic fungus that causes anthracnose in various plants, including tomatoes, eggplant, papaya, mango, chili peppers, and others. This disease is a

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major horticultural problem in Indonesia and can significantly reduce both the quantity and quality of harvests. Currently, anthracnose is primarily managed using synthetic chemical fungicides. Although these fungicides are effective, their long-term use poses negative impacts on the environment and human health and can lead to the development of pathogen resistance (Pratama et al., 2025).

Pestalotiopsis sp. is a weak parasitic pathogen that infects wounds. The wind carries fungal spores (conidia). Over short distances, the spores can be carried by splashing water and insects. Consequently, it spreads very rapidly and causes continuous leaf drop. The spread of Pestalotiopsis sp. in rubber plants begins with direct leaf infection or through wounds. The pathogen then develops in the parenchymal tissue, settling and developing in the vascular bundles. The morphology of Pestalotiopsis sp. is characterized by a colony color that changes with age: initially white, then black spots appear over time, and the base of the colony becomes brownish-yellow (Manik, 2022).

Phytophthora sp. is a group of oomycete pathogens widely known as the primary cause of various plant diseases in horticulture, forestry, and tropical plantations. Diseases caused by Phytophthora spp. include root, stem, and fruit rot. Phytophthora sp. As the primary pathogen, measures can be taken to inhibit its growth in plants, for example, by removing or cutting diseased plant organs and using synthetic pesticides. Stem cancer control efforts should focus on effectively suppressing the pathogenic fungus Phytophthora sp. (Abdilla et al., 2025).

Trichoderma sp. is a naturally occurring soil saprophytic fungus that attacks many types of plant disease-causing fungi and provides broad-spectrum control. Trichoderma sp. can hyperparasitize several types of plant disease-causing fungi and grows very rapidly. The advantages of using Trichoderma sp. as a potential biological agent are its rapid growth and ease of cultivation in culture and natural conditions (Nurhajijah et al., 2025; Berlian et al., 2013). Trichoderma sp. is a saprophytic fungus because it exhibits antagonistic properties against pathogens, including competition for space and nutrients, microparasitism, and antibiosis. It also has several advantages, such as easy isolation, broad adaptability, ubiquitous presence in soils of cultivated areas, rapid growth on various substrates, broad mycoparasitism, and non-pathogenicity to plants (Utami et al., 2021). Because Trichoderma sp. has strong antagonistic capabilities, research is needed to determine its inhibitory efficacy against Colletotrichum sp., the causal agent of anthracnose disease (Halifu et al., 2020; Fardhani et al., 2023).

The dual-culture assay method is widely used in vitro to evaluate the antagonistic activity of Trichoderma sp. against various plant pathogens. This experimental approach has been widely used in modern biocontrol research to assess the interaction between antagonists and pathogens. Dual-culture assays are conducted to determine the ability of Trichoderma sp. to parasitize, compete for space and nutrients, and cause antibiosis and lysis (Manzar et al., 2022; Dewi et al., 2015). The mycoparasitic mechanism is demonstrated by antagonist fungal hyphae attaching to, entangling, or penetrating pathogenic fungal hyphae, causing them to flatten and lyse (Adhikari et al., 2023; Rahmawati et al., 2018).

The split plate method is an in vitro technique used to evaluate the volatile antibiosis activity of antagonistic microorganisms such as Trichoderma sp. against plant pathogens. The use of antagonistic microorganisms as biological control agents can suppress attacks that cause plant damage without harming plants or the environment (Muhibuddin et al., 2024). This technique is designed to physically separate antagonist and pathogen colonies in a single petri dish, preventing direct contact between hyphae. Separation is achieved by using a partition or by placing each isolate in two opposing media sections separated by an air space in the dish. The main objective of this method is to assess the extent to which volatile organic compounds (VOCs) produced by Trichoderma can inhibit the growth of airborne pathogens without relying on mycoparasitism mechanisms or direct nutrient competition. Volatile organic compounds (VOCs) are among the most common organic pollutants in water. These compounds have been detected in natural waters, wastewater, and drinking water (Yamindago et al., 2024).

The split plate test typically begins with preparing two solid media, such as potato dextrose agar (PDA), and placing them separately on opposite sides of a petri dish. Trichoderma isolates are inoculated on one side, while the test pathogen (e.g., Colletotrichum, Phytophthora, or Pestalotiopsis) is inoculated on the other. The two media are then combined in a tightly sealed petri dish, allowing the air inside to serve as a medium for the diffusion of volatile compounds. The dish is incubated at a constant temperature (generally 25–28°C) for several days. After the incubation period, the radial growth of the pathogen is measured to calculate the percentage of mycelial growth inhibition (PIRG) compared to a control without Trichoderma. This study aims to evaluate the antagonistic potential of Trichoderma reesei against three major tropical plant pathogens − Colletotrichum gloeosporioides, Pestalotiopsis microspora, and Phytophthora palmivora − using both dual-culture and split-plate techniques.

2. Material and Methods

This research was conducted at the Microbial Taxonomy and Agricultural Quarantine Laboratory of the Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, Malaysia, at coordinates 2559 18.1" North Latitude 101°42'5.8" East Longitude, at an altitude of 36 meters above sea level

(masl). This research was conducted in September 2025.

This research was conducted from September 21, 2025, to October 10, 2025, beginning with media preparation and tub culture preparation.

The materials used were Potato Dextrose Agar (PDA), Trichoderma isolates (test cultures), and plant pathogen isolates such as Colletotrichum, Pestalotiopsis, and Phytophthora.

Tools used: Petri dishes, plastic/agar dividers, pipettes, inoculation loops, spatulas, autoclaves, laminar flow cabinets, incubators set at the appropriate temperature, tissue paper, 70% alcohol, markers, rulers/vernier calipers.

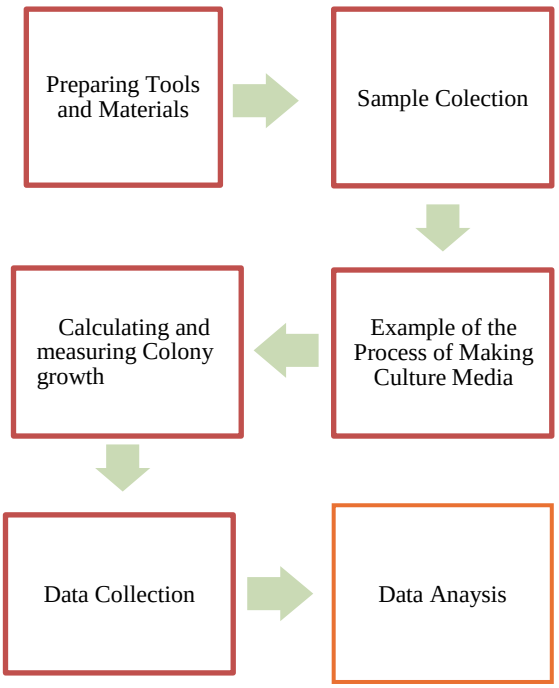

Figure 1. Research flow diagram

The dual culture method will be implemented by placing the mycelial disks of Colletotrichum gloeosporioides, Pestalotiopsis spp., and Phytophthora palmivora pathogens at a distance of 1.5 cm from the center of the PDA plate, and the mycelial disk of Trichoderma spp. will be placed 3 cm from the mycelial disk of Colletotrichum gloeosporioides, Pestalotiopsis spp., and Phytophthora palmivora. All plates will then be incubated at 28 °C for 7 days. Pathogens will serve as controls in the absence of Trichoderma on the PDA plate. All dual-culture tests will be performed in 4 replicates and incubated at 28 °C for 7 days. The results of the observations will be used to determine the pathogen's response to Trichoderma spp. The diameter of the Trichoderma spp. colony. which inhibits the growth of Colletotrichum gloeosporioides, Pestalotiopsis spp., and Phytophthora palmivora in the dual culture test will be used in the calculation of the Percentage of Radial Growth Inhibition (PIRG) with the following formula:

$$\%PIRG = (R_1 - R_2 \ X\ 100)/R_1$$

R_1 indicates radial growth of Trichoderma in the absence of an antagonist (control plate), while R_2 indicates radial growth of Trichoderma in the presence of an antagonist on a double culture plate.

The split plate method involves inoculating one plate with Trichoderma (a 5 mm diameter plug placed in the center). Incubate for 48 hours at 25°C to allow for volatile compound (VOC) production. Inoculate another separate plate with the pathogens Colletotrichum gloeosporioides, Pestalotiopsis spp., and Phytophthora palmivora (a 5 mm diameter plug placed in the center). In a laminar flow cabinet, remove the lids of both plates and position them facing each other (sides facing each other without touching). Seal both plates tightly with parafilm to prevent air exchange. All split plates will be prepared in four replicates and incubated at 25°C for 5–7 days. Measure the radial growth (colony diameter in mm) of the pathogen every 24 hours for up to 7 days (daily). Record colony morphology, sporulation, and any abnormalities compared to the control. Document the culture by photographing each observation point. The percentage inhibition of pathogen growth is calculated as:

$$\%Inhibition = (R_{control} - R_{treatment} \ X\ 100)/R_{control}$$

where $R_{control}$ is the average diameter of the pathogen colonies in the control treatment, and $R_{treatment}$ is the diameter of the pathogen colonies in the presence of VOCs produced by Trichoderma.

Data were analyzed using analysis of variance (ANOVA) with the General Linear Model (GLM) procedure in SAS software (SAS Institute Inc., Cary, NC, USA). The factor tested was pathogen type, and mean comparisons were performed using the Least Significant Difference (LSD) test at a significance level of $p \leq 0.05$. Data collection required observational diameter calculations over 7 days. After collection, the data were entered into Microsoft Excel, containing the observation data from the dual culture test and split plate methods. After data collection, the data were entered into the statistical software program SAS (Statistical Analysis System). This began with the preparation of raw data obtained from laboratory measurements, such as colony growth diameter, percentage inhibition of radial growth, or other parameters. The data was compiled in tabular format using Microsoft Excel or a CSV file before being imported into SAS.

3. Results and Discussion

3.1. Double Culture

The inhibitory effect of T. reesei against the three tested pathogens showed significant differences ($F_{2,9}$ = 9.38; p = 0.0063; R^2 = 0.68), indicating that the pathogen type factor explained 68% of the variation in inhibition. The highest inhibition was recorded against P. microspora (89.37 ± 1.48%), followed by C. gloeosporioides (87.22 ± 2.01%), and the lowest against P. palmivora (84.37 ±

1.35%) (Table 1; Figure 1). The LSD test ($p \leq 0.05$) showed that the inhibition against Pestalotiopsis sp. and C. gloeosporioides was not significantly different, but both were significantly higher than against P. palmivora. T. reesei showed strong antagonism against all pathogens, although the level of inhibition against P. palmivora was slightly lower. According to Elfina et al. (2024) and Widyaningsih & Triasih (2021), the percentage of inhibition of Pestalotiopsis sp. fungal growth also increases. This can be linked to the growth of Pestalotiopsis sp. fungal colonies, which is influenced by the concentration of chitosan given. The smaller diameter of Pestalotiopsis sp. fungal colonies indicates that there has been inhibition of fungal growth. Trichoderma's ability to control various pathogens is due to several mechanisms, including: producing enzymes, inducing plant resistance, being antagonistic (mycoparasitism, antibiosis, competition), increasing nutrient availability, and inactivating pathogen enzymes (Adhikari et al., 2023; Molebila et al., 2020).

During the four-day incubation period, the inhibition rate increased progressively (Figure 2). Trichoderma inhibition against Colletotrichum gloeosporioides in the split plate system reached only 20 – 30%, much lower than in the dual culture test. In addition to stimulating plant growth and providing nutrients, actinomycetes act as control agents for soil-derived pathogens or as bioprotectants by producing various anti-pathogenic compounds and metabolites, such as siderophores, β-1,3-glucanase, chitinase, antibiotics, and cyanide. The antagonistic mechanisms that often occur are parasitism, antibiosis, lysis, and competition (Loc et al., 2020). Among the three pathogens, T. reesei showed the strongest antagonistic effect against C. gloeosporioides, followed by Pestalotiopsis sp. and P. palmivora. The gradual increase in inhibition indicates that the antagonistic activity of T. reesei is time-dependent and is primarily controlled by direct interactions between hyphae and overgrowth, reflecting its competitive and mycoparasitic abilities (Figure 2).

Table 1. Average percentage of pathogen inhibition by Trichoderma reesei in dual culture test (day 4).

Pathogen	Mean Inhibition (%) ± SD
Pestalotiopsis microspora	89.37 ± 1.48 a
Colletotrichum gloeosporioides	87.22 ± 2.01 a
Phytophthora palmivora	84.37 ± 1.35 b

Means followed by the same letter are not significantly different at the $p \leq 0.05$ level (LSD test).

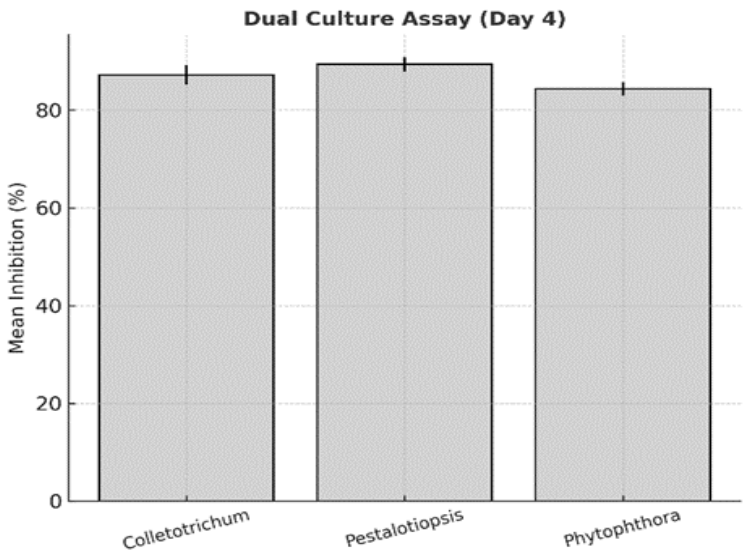

Figure 2. Mean percentage inhibition (± SD) of C. gloeosporioides, P. microspora, and P. palmivora by Trichoderma reesei in the dual culture test (day 4). Different letters above the bars indicate significant differences based on the LSD test at the $p < 0.05$ level.

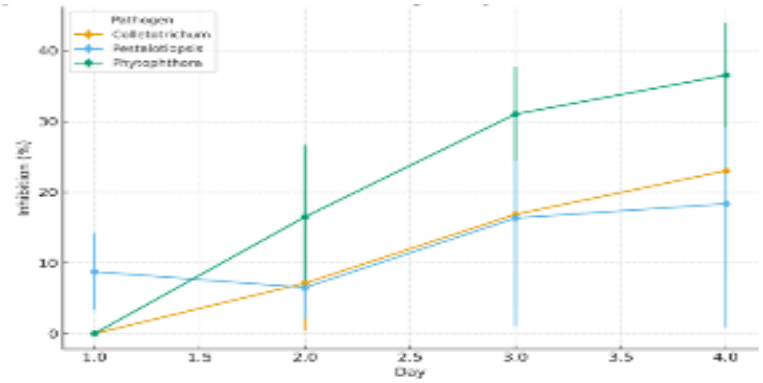

Figure 2. Daily growth inhibition percentage of three pathogens (P. palmivora, C. gloeosporioides, and P. microspora) in a dual culture test with Trichoderma reesei. Values shown are the mean ± standard deviation (SD) for each observation day.

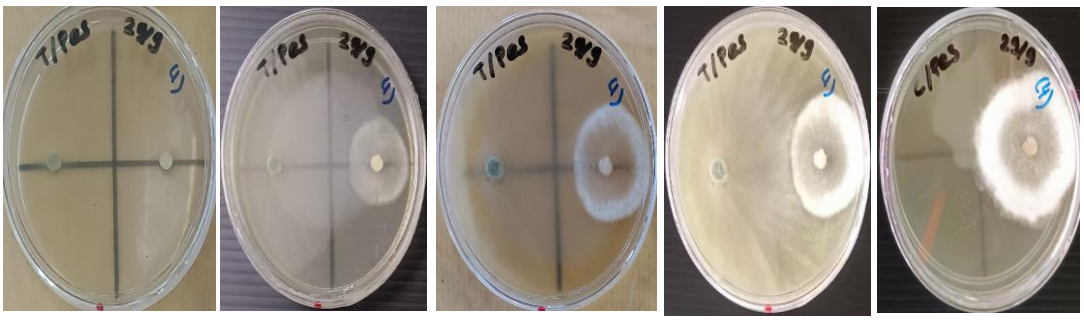

Figure 3. Potential antagonist test for Pestalotiopsis pathogen growth. Far left to right: Pestalotiopsis pathogen growth on days 1-4. Far Right: Control

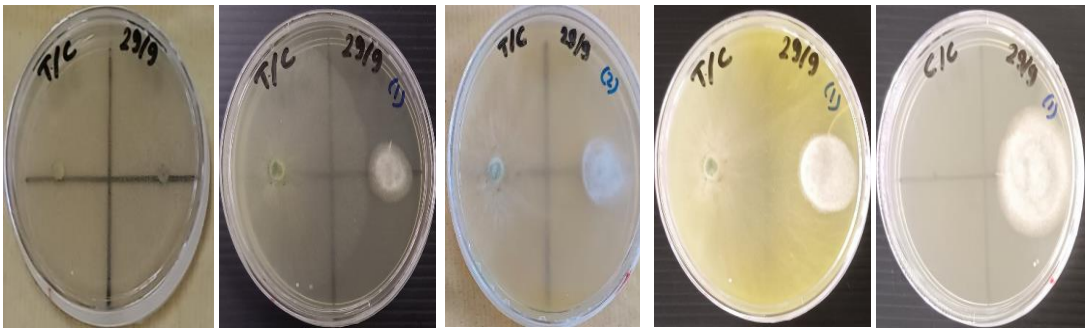

Figure 4. Potential antagonist test for Colletotricum pathogen growth. Far left to right: Colletotricum pathogen growth on days 1-4. Far Right: Control

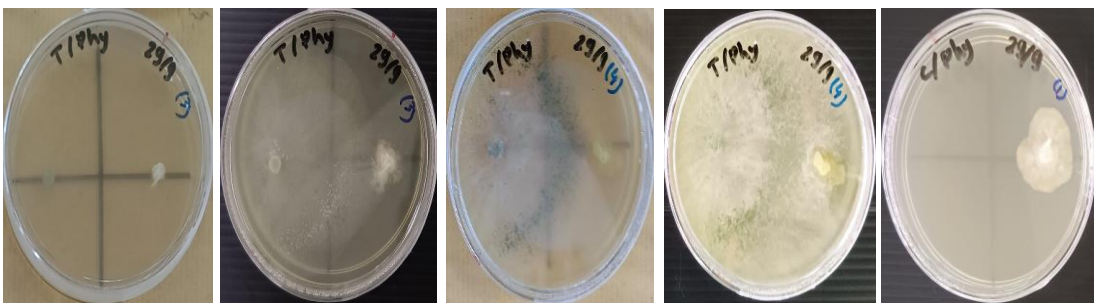

Figure 5. Potential antagonist test for Phytophthora pathogen growth. Far left to right: Phytophthora pathogen growth on days 1-4. Far Right: Control

3.2. Split Plate

In the split plate test, the inhibitory activity of Trichoderma reesei against the three test pathogens was generally lower than the results in the dual culture test (Table 2). The average inhibition percentages against Colletotrichum gloeosporioides, Pestalotiopsis microspora, and Phytophthora palmivora were 22.9 ± 11.1%, 16.7 ± 16.2%, and 8.7 ± 6.1%, respectively (Figure 2). Although T. reesei showed a numerically higher inhibition value against C. gloeosporioides, the ANOVA analysis showed no significant differences among the three pathogens ($F_{2,9}$ = 1.43; p = 0.288; R^2 = 0.24). The LSD test (LSD = 18.97) also showed that all mean values were included in the same significance group (p > 0.05). The inhibition pattern in the split plate test showed a similar trend to the results in the dual culture test, indicating that volatile compounds produced by T. reesei also play a role in suppressing pathogen growth. The antagonism test method for determining the ability of biocontrol agents to produce volatile compounds that act as antifungals involves growing the biocontrol agent in a medium in a Petri dish that differs from the pathogen's medium (Benatar et al., 2025). The level of effectiveness is lower than that of the mechanism involving direct contact between hyphae. In the mechanism of antagonism, Trichoderma inhibits spore germination and disrupts the pathogen's metabolic processes. The results of this study indicate that volatile metabolites produced by T. reesei exert a moderate inhibitory effect, but their effectiveness is lower than that observed with direct interaction in the dual culture test. This indicates that the more dominant inhibitory mechanism comes from the secretion of non-volatile metabolites, enzymatic activity, and the growth of hyphae that cover the pathogen.

Table 2. Average percentage of pathogen inhibition by Trichoderma reesei in the split plate test (day 7).

Pathogen	Mean ± SD (%)
Colletotrichum gloeosporioides	22.9 ± 11.1a
Pestalotiopsis microspora	16.7 ± 16.2a
Phytophthora palmivora	8.7 ± 6.1b

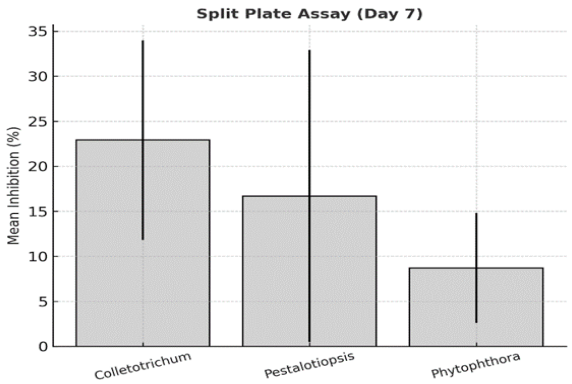

Figure 6. Average percentage inhibition (± SD) of Colletotrichum gloeosporioides, Pestalotiopsis sp., and Phytophthora palmivora by Trichoderma reesei in the split plate test (day 7). There was no significant difference between treatments (p > 0.05).

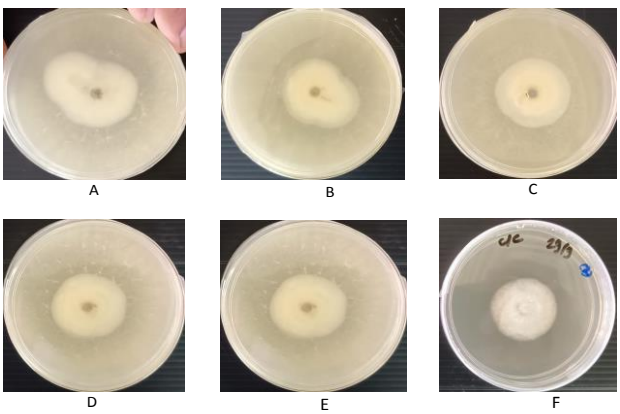

Figure 7. Potential Antagonist Test for Colletotrichum Pathogen Growth. A-D. Growth of Colletotrichum pathogen on days 1 to 4. E. Growth of Colletotrichum pathogen on day 7. F. Control

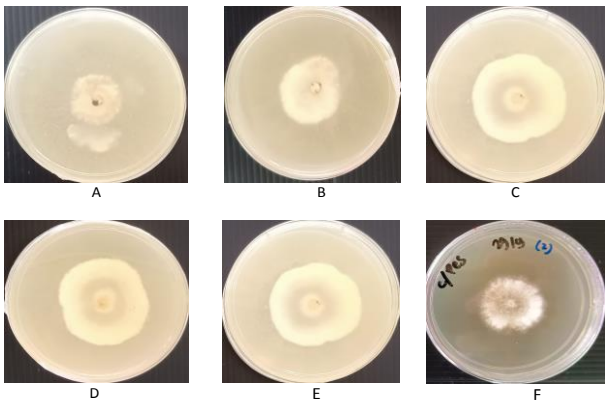

Figure 8. Potential Antagonist Test for Pestalotiopsis Pathogen Growth. A-D. Growth of Colletotrichum pathogen on days 1 to 4. E. Growth of Pestalotiopsis pathogen on day 7. F. Control

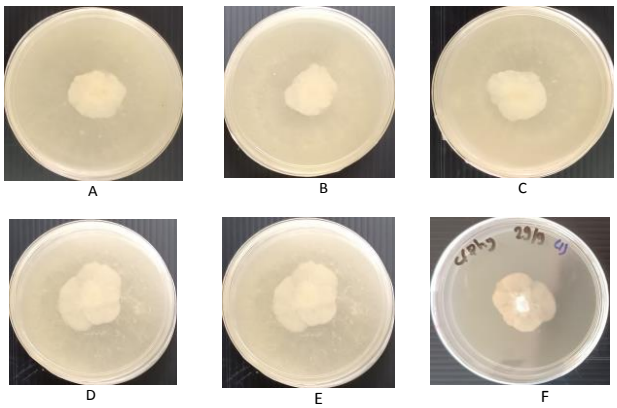

Figure 9. Potential Antagonist Test for Phytophthora Pathogen Growth. A-D. Growth of Colletotrichum pathogen on days 1 to 4. E. Growth of Phytophthora pathogen on day 7. F. Control

The dual culture assay emphasizes direct interactions between Trichoderma and the target pathogen, allowing for various mechanisms such as hyphal contact, coiling, enzymatic degradation, and growth over the pathogen colony. In contrast, the split plate assay eliminates physical contact and primarily evaluates indirect inhibition mediated by volatile organic compounds (VOCs). The percentage inhibition obtained from the split plate assay was lower than that from the dual culture assay, but both showed a consistent ranking pattern (C. gloeosporioides > P. microspora > P. palmivora). Volatile compounds exhibit strong inhibitory activity against plant pathogenic fungi (Suryadi et al., 2015). This parallel pattern suggests that T. reesei possesses a broad spectrum of antagonistic mechanisms, capable of suppressing pathogens through both contact-dependent and volatile compound-mediated interactions.

The relatively weaker inhibition in the split plate assay indicates that volatile metabolites are less effective when acting singly. The measurable suppression of pathogen growth indicates that VOCs produced by T. reesei, such as 6-pentyl-α-pyrone, play a significant role in its antagonistic potential. Trichoderma inhibition of Phytophthora palmivora in the split plate system reached only 5 – 10%, whereas in the dual culture test, the low inhibition value of 8.7 ± 6.1% indicates that Trichoderma's volatile antibiosis mechanism plays a very limited role against oomycete pathogens such as Phytophthora. These volatile compounds are known to disrupt fungal membrane integrity and inhibit mycelial development. Trichoderma inhibition of Pestalotiopsis sp. in the split plate system only reached 10–25%, while in the dual culture test, it could exceed 80%. According to Adhikari et al. (2023), differences in the percentage of inhibition between isolates are due to the different bacterial isolates tested, resulting in varying inhibition of metabolite activity. The low inhibition of Pestalotiopsis in the split plate test indicates that the effectiveness of Trichoderma biocontrol depends on a combination of direct and non-contact mechanisms. Fungi of the Trichoderma genus, which use competition and parasitism mechanisms, generally have a broader and stronger inhibitory spectrum, thereby preventing the growth of pathogens. The parasitic activity of Trichoderma antagonistic fungi produces toxic chemical compounds and enzymes that degrade pathogen cells (Ruangwong et al., 2021; Rajani et al., 2021).

4. Conclusion

Trichoderma reesei exhibits strong antagonistic potential against several major tropical plant pathogens.

This research offers a valuable scientific contribution to sustainable plant disease management strategies. Environmental factors such as humidity, temperature, and soil pH should be considered, as they can significantly influence T. reesei activity. Optimizing these conditions will enhance the colonization and antagonistic effectiveness of this microorganism in the field.

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