



## TEST POTENTIAL OF SELECTED BIOLOGICAL CONTROL FUNGI AGAINST *Phyllosticta Zingiberi* UNDER LABORATORY CONDITIONS

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### ABSTRACT

Leaf spot disease is one of the major constraints in red ginger (*Zingiber officinale* Rosch) cultivation, causing necrosis on leaves that reduces photosynthetic capacity and rhizome productivity. This study evaluated the antagonistic activity of four biological control agents *Trichoderma koningii*, *Trichoderma harzianum*, *Gliocladium* spp., and *Gliocladium virens*, against *Phyllosticta zingiberi* under *in vitro* conditions. The experiment was conducted at the Plant Pathology Laboratory, Faculty of Agriculture, University of Sumatera Utara, Medan, from January to March 2010. A Completely Randomized Design (CRD) with five treatments and five replications was used. The results showed that the highest growth inhibition was observed in *Gliocladium* spp. (46.23%) and *T. harzianum* (46.15%), followed by *T. koningii* (41.38%) and *G. virens* (27.24%). The control exhibited the lowest inhibition (6.43%). The observed suppression is attributed to mechanisms including mycoparasitism, production of antifungal metabolites, and competition for space and nutrients. The findings indicate that *Gliocladium* spp. and *T. harzianum* are the most effective biological control agents for managing leaf spot disease in red ginger.

**Keywords:** *Phyllosticta zingiberi*, *Trichoderma koningii*, *Trichoderma harzianum*, *Gliocladium* spp., *Gliocladium virens*

### INTRODUCTION

Red ginger (*Zingiber officinale* Rosch) is a high-value horticultural commodity widely cultivated for its culinary, medicinal, and economic importance, including substantial contributions to export markets. Production of red ginger, however, is constrained by biotic stresses, notably leaf spot disease caused by *Phyllosticta zingiberi*, which leads to necrotic lesions on leaves that reduce the effective photosynthetic area and consequently decrease plant growth and rhizome yield. *Phyllosticta zingiberi* has been reported as a dominant cause of leaf spot in ginger in several production regions and poses a risk to yield stability if not managed properly. (Annisa Khoiriyah, Heriyanto, and Fitria Naimatu Sa'diyah, 2023) Current control strategies for leaf spot in ginger rely heavily on synthetic fungicides and amendments such as foliar fertilizers, but these approaches have limitations. While fungicides can suppress disease intensity, their repeated application can lead to fungicide resistance in pathogen populations, residual chemicals in produce, environmental contamination, and negative impacts on beneficial soil microflora. Integrated approaches combining fungicide use with nutrient management have been explored, yet concerns regarding sustainability and environmental safety remain. (Annisa Khoiriyah, Heriyanto, and Fitria Naimatu Sa'diyah, 2023)

Biological control through the use of antagonistic microorganisms such as *Trichoderma* spp. and *Gliocladium* spp. offers a more environmentally benign alternative. These

organisms exert antagonism via several mechanisms, including mycoparasitism, competition for nutrients and space, production of antifungal metabolites, and induction of host plant defenses. While studies have documented the use of *Trichoderma*-based biopesticides to reduce disease severity in ginger caused by *P. zingiberi*, with formulated biopesticide combinations showing measurable suppression of disease progression, available evidence suggests the need for further systematic evaluation of specific antagonists under controlled conditions. (Laila Rahma Munawaroh, 2023)

Despite the documented potential of biological control agents in reducing disease incidence, research specifically addressing the *in vitro* antagonistic interactions between multiple biocontrol fungi (*Trichoderma koningii*, *Trichoderma harzianum*, *Gliocladium spp.*, *Gliocladium virens*) and *P. zingiberi* remains limited. Establishing the inhibitory efficacy of these agents under laboratory conditions is essential as a foundational step toward developing sustainable disease management protocols that can be further validated in greenhouse and field trials. (Annisa Khoiriyah, Heriyanto, and Fitria Naimatu Sa'diyah, 2023)

## **MATERIALS AND METHODS**

### **Study Site and Duration**

The research was conducted at the Plant Pathology Laboratory, Faculty of Agriculture, Universitas Sumatera Utara, Medan, Indonesia, located at an altitude of approximately 25 meters above sea level, from January to March, 2010.

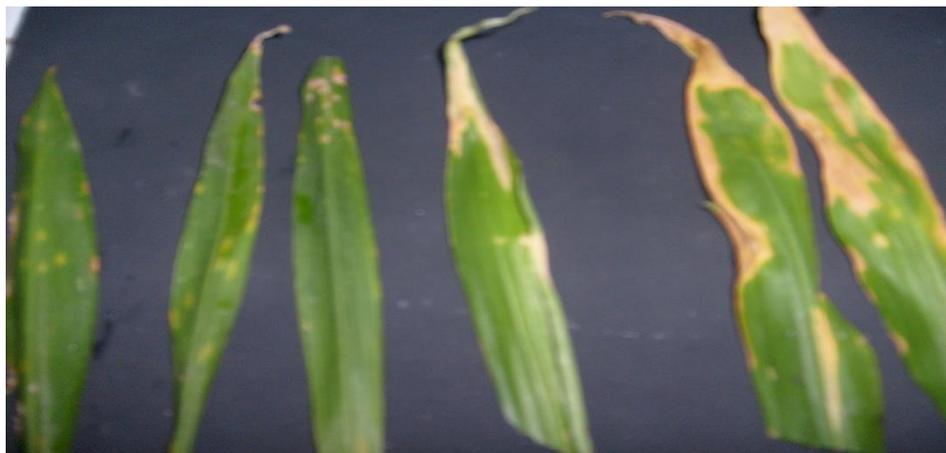
### **Materials and Equipment**

The materials used in this study included the following: the isolates of *Trichoderma koningii* and *Trichoderma harzianum* were obtained from the Seed and Plantation Crop Protection Center, Medan, Indonesia. The *Gliocladium spp.* isolate was collected from the Fruit Crop Experimental Station, Berastagi, Indonesia, while the *Gliocladium virens* isolate was obtained from the Indonesian Center for Plant Pest Forecasting (BBPOPT), Jatisari, Karawang, West Java, Indonesia. The materials used in this study included 96% ethanol, distilled water, dextrose, and healthy red ginger plants. The equipment used consisted of Petri dishes, test tubes, Erlenmeyer flasks, graduated pipettes, glass slides, an analytical balance, inoculation needles, a cork borer, forceps, polybags, a microscope, an autoclave, an oven, sterile cotton, a calculator, a writing board, labeling paper, buckets, scissors, and a hand sprayer.

### **Experimental Procedures**

The pathogen inoculation procedure was initiated by collecting several leaves of red ginger plants showing typical leaf spot symptoms (Figure 1). The pathogen *Phyllosticta zingiberi* was isolated from symptomatic red ginger leaves showing typical leaf spot lesions. Infected leaf tissues were surface sterilized in 1% sodium hypochlorite solution for 1–2 minutes, rinsed three times with sterile distilled water, air-dried under aseptic conditions, and plated on Potato Dextrose Agar (PDA). The plates were incubated at  $27 \pm 2^\circ\text{C}$  for 5–7 days. Pure cultures were obtained using the hyphal tip method and maintained on PDA slants for further experiments.

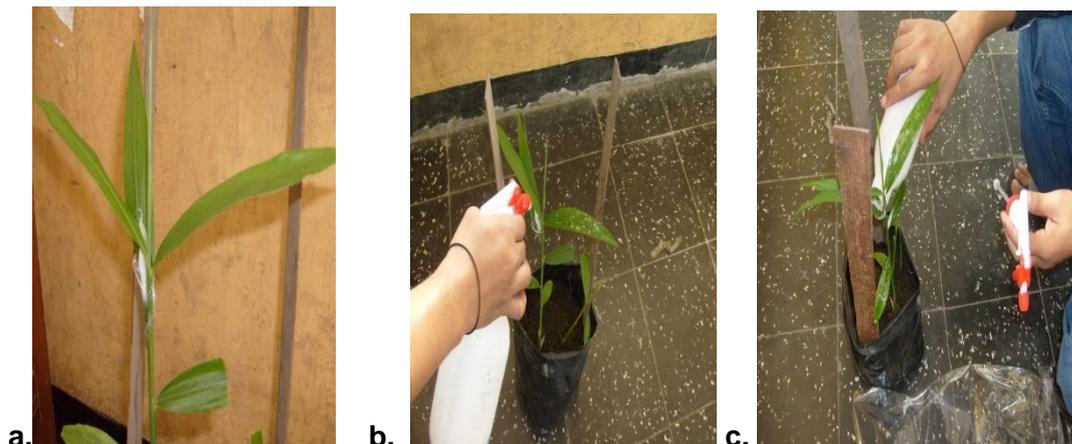
The biological control agents used in this study were *Trichoderma koningii*, *Trichoderma harzianum*, *Gliocladium spp.*, and *Gliocladium virens*. Each isolate was cultured on PDA medium and incubated at  $27 \pm 2^\circ\text{C}$  for 5–7 days prior to antagonistic assays.



**Figure 1.** Red ginger leaves exhibiting typical leaf spot symptoms were collected and used as samples for pathogen isolation.

Morphological identification of *P. zingiberi* was conducted based on colony characteristics, conidial morphology, and microscopic observation using standard taxonomic keys. Pathogenicity was confirmed through Koch's postulates by inoculating healthy red ginger leaves with a conidial suspension of the pathogen, followed by re-isolation of the fungus from resulting lesions. In this experiment, pathogen inoculation was performed using three different methods: Method 1. Direct attachment of *Phyllosticta zingiberi* mycelium onto the leaf surface. Healthy ginger leaves were gently wounded using fine sandpaper prior to inoculation. A square agar plug (approximately 1 cm in diameter) containing actively growing mycelium of *P. zingiberi* was cut from a Petri dish culture and placed onto the wounded area of the leaf. The inoculated site was covered with sterile moist cotton and secured with cling wrap to maintain humidity (Figure 2a). Method 2. Foliar spray inoculation with fungal suspension. A fungal suspension was prepared by scraping the fungal isolate from a Petri dish until the mycelium was released, then transferring it into a hand sprayer containing 200 mL of sterile distilled water. A small amount of detergent was added as a surfactant to ensure even distribution. The suspension was sprayed onto the surface of healthy ginger leaves until runoff. The plants were then covered with transparent plastic to maintain high humidity. The date of Koch's postulate inoculation was recorded, and symptom development was observed daily (Figure 2b). Method 3. Soil spray inoculation with fungal suspension. A fungal suspension was prepared as described above by macerating the fungal culture and suspending it in 200 mL of sterile distilled water with a small amount of detergent as a surfactant. The suspension was sprayed onto the soil surface of healthy red ginger plants. The plants were subsequently covered with transparent plastic to maintain humidity. The inoculation date for Koch's postulate verification was recorded, and disease development was monitored daily (Figure 2c).

The antagonistic activity of biological control agents against *P. zingiberi* was evaluated using the dual culture method on PDA medium. A 5-mm diameter mycelial disc of the pathogen was placed on one side of a Petri dish containing PDA, while a 5-mm disc of the antagonist was placed on the opposite side at an equal distance from the center.



**Figure 2.** (a) Method 1: The pathogen was directly attached to the leaf surface; (b) Method 2: The pathogen suspension was sprayed onto the leaf surface; (c) Method 3: The pathogen suspension was sprayed onto the soil surface.

Control plates consisted of the pathogen cultured alone on PDA. Each treatment was replicated four times in a completely randomized design (CRD). All plates were incubated at  $27 \pm 2^{\circ}\text{C}$  for 7 days. Radial growth of the pathogen colony was measured daily until the control plate reached the edge of the Petri dish. Following the completion of Koch's postulates, the fungus was subjected to further identification to confirm the causal agent of the disease using relevant taxonomic literature as references. Fungal identification was performed based on both macroscopic and microscopic morphological characteristics. Colonies grown on pure culture media were examined for colony morphology, including color, texture, and growth pattern, and subsequently observed under a microscope to assess conidial shape, size, and other diagnostic structures. Leaf tissues exhibiting symptoms that developed during the Koch's postulate test were re-isolated to verify that the recovered fungus was identical to *Phyllosticta zingiberi*, thereby confirming its role as the causal pathogen of the observed leaf spot disease.

### Research Methods

The antagonistic assay was conducted when the fungal cultures reached six days of incubation. The experiment was arranged in a completely randomized design (CRD) with a non-factorial treatment structure, consisting of five treatments and five replications. The treatments were as follows:

A = control (without antagonistic fungus);

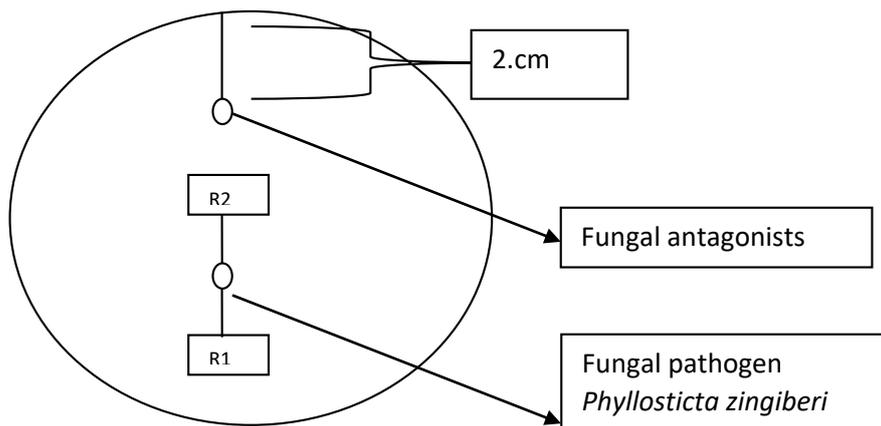
K = *Trichoderma koningii*;

H = *Trichoderma harzianum*;

G = *Gliocladium* spp.;

V = *Gliocladium virens*.

The objective of this assay was to evaluate the antagonistic capacity and percentage inhibition of the biological control agents *Trichoderma koningii*, *Trichoderma harzianum*, *Gliocladium* spp., and *Gliocladium virens* against the fungal pathogen *Phyllosticta zingiberi*. The antagonistic interaction between the biocontrol agents and the pathogen was quantitatively assessed based on radial growth inhibition. A schematic representation of the antagonistic assay is presented in Figure 3.



**Figure 3.** Schematic diagram of the antagonistic assay on Potato Dextrose Agar (PDA) medium.

The inhibition of *Phyllosticta zingiberi* growth by the biological control agents was calculated using the following formula:

$$\text{Percentage Inhibition of Radial Growth (PIRG)} = P = \frac{R_1 - R_2}{R_1} \times 100\%$$

where:

$R_1$  = radial growth of the pathogen in the control treatment (mm)

$R_2$  = radial growth of the pathogen in the presence of the antagonistic fungus (mm).

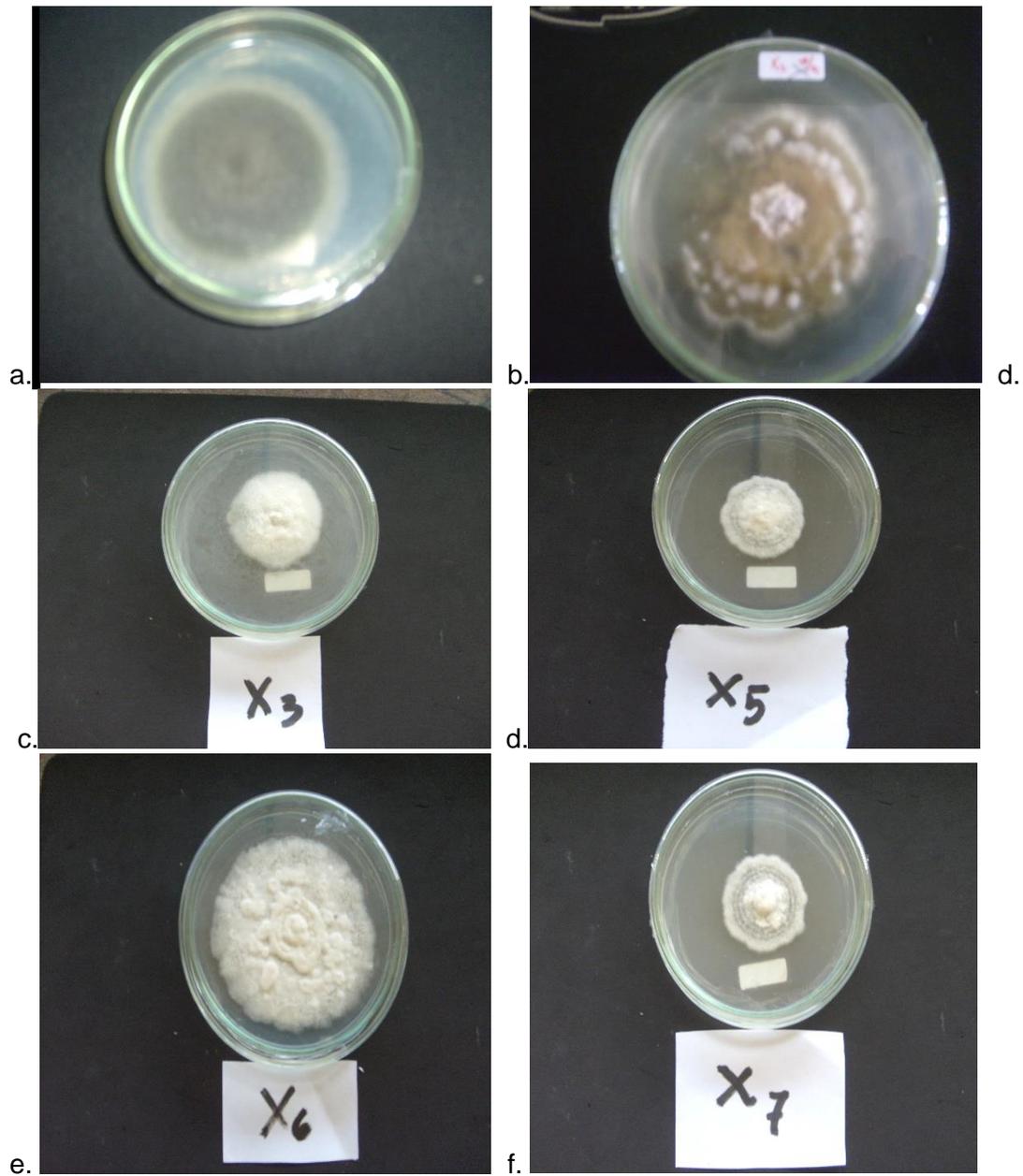
(Skidmore and Dickinson, 1976, as cited in BPTPH, 2002).

Data analysis of variance (ANOVA) revealed highly significant differences among treatments. To determine which treatments differed significantly, Duncan's Multiple Range Test (DMRT)

## RESULTS AND DISCUSSION

### Isolation of the Causal Pathogen of Ginger Leaf Spot Disease

The results of pathogen isolation indicated that among the isolates obtained from samples X1 to X7, only certain samples were morphologically consistent with *Phyllosticta zingiberi*. Microscopic observations revealed that the conidia exhibited morphological characteristics typical of *P. zingiberi*, as shown in Figure 4. Recent studies on the causal agent of ginger leaf spot have further elucidated the cultural and physiological behavior of *Phyllosticta zingiberi*, including optimal growth conditions and morphological features observed in vitro. Sampritha et al. (2024) reported that *P. zingiberi* exhibited maximum mycelial growth at specific temperatures and pH levels, which supports phenotypic descriptions obtained from pure isolates in this study. Morphological characterization of the pathogen by Rai et al. (2017) provided detailed measurements of pycnidia and spore morphology, corroborating classical descriptions used for identification in this research. Furthermore, field studies have confirmed that *P. zingiberi* remains a significant disease constraint in ginger cultivation, with recent work demonstrating that appropriate fungicide and nutrient management can influence disease severity and yield outcomes.



**Figure 4.** (a) Colony morphology of *Phyllosticta zingiberi* isolates on PDA medium: (a) X1, 3 days; (b) X2, 3 days; (c) X3, 3 days; (d) X5, 3 days; (e) X6, 5 days; (f) X7, 3 days after incubation.



**Figure 5.** Microscopic morphology of *Phyllosticta zingiberi* conidia observed at the Plant Pathology Laboratory, Faculty of Agriculture, Universitas Sumatera Utara (January 28, 2010).

### Koch's Postulates Test

The results of Koch's postulates test showed that the disease symptoms observed in the inoculated plants were consistent with those found under field conditions (Figure 6a and Figure 6b).



**Figure 6.** (a) Ginger plant naturally infected with leaf spot disease collected from the research field of; (b) Ginger plant exhibiting leaf spot symptoms following Koch's postulates test (Martha, 2010).

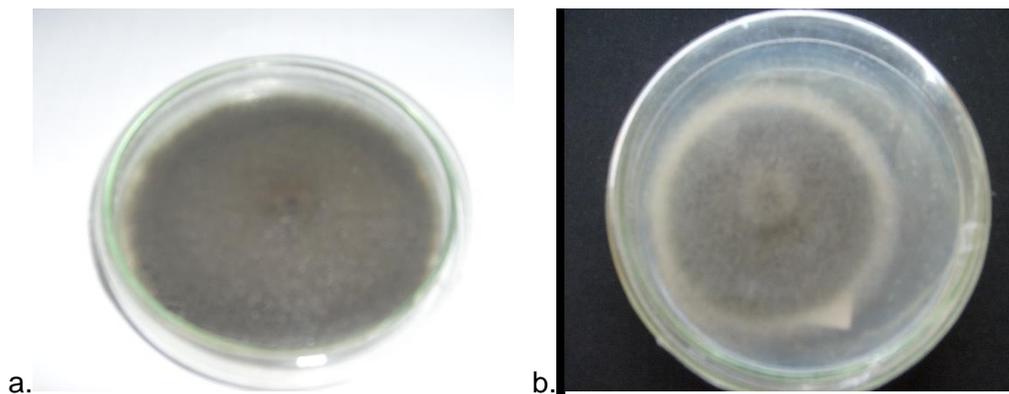
Leaves exhibiting leaf spot symptoms following Koch's postulates test (Figure 7) were re-isolated to confirm whether the pathogen obtained after the pathogenicity test was identical to the original pure culture of *Phyllosticta zingiberi* isolated from field samples.



**Figure 7.** Ginger leaf showing leaf spot symptoms after inoculation and completion of Koch's postulates verification (Martha, 2010).

### Identification of *Phyllosticta zingiberi*, the Causal Agent of Ginger Leaf Spot Disease

The results indicated that the fungal isolates obtained from pure cultures of *Phyllosticta zingiberi* and those re-isolated after Koch's postulates exhibited similar morphological characteristics. Colonies grown on Potato Dextrose Agar (PDA) appeared gray to dark brown with diffuse margins, consistent with descriptions of *P. zingiberi* morphology in recent studies. The comparison of fungal growth on PDA medium is shown in Figure 8.



**Figure 8.** (a) Pure culture of *Phyllosticta zingiberi*; (b) Pure culture re-isolated after Koch's postulates.

Morphological features such as colony pigmentation and marginal appearance are commonly used as initial diagnostic criteria for species identification in *Phyllosticta spp.*, although they can show some variability depending on culture conditions and incubation time. For example, Sampritha *et al.* (2024) reported that *P. zingiberi* isolates consistently produced robust mycelial growth on PDA and other media with characteristic coloration patterns under controlled incubation conditions, reinforcing the reliability of morphological observations for preliminary identification. The observed morphological concordance between field isolates and re-isolated cultures after Koch's postulates supports the fulfillment of pathogenicity criteria and confirms the causal role of *P. zingiberi* in the leaf spot symptoms. This approach aligns with standard pathogen identification protocols, in which re-isolation and comparison of morphological traits provide evidence of identity and pathogenic consistency. Similar methodologies have been adopted in other recent plant pathology research, where morphological and cultural characteristics are integrated with pathogenicity tests to validate causal agents. Although morphological identification remains fundamental, it is widely acknowledged in contemporary phytopathological research that morphological traits should ideally be complemented with molecular tools (ITS sequencing) for more definitive species confirmation, particularly among closely related fungal taxa. This reflects a trend in recent studies of fungal leaf spot diseases, where molecular data are used alongside morphology to resolve species boundaries and strengthen diagnostic accuracy.

### Test Potential Of Selected Biological Control Fungi Against *Phyllosticta Zingiberi* Under Laboratory Conditions

#### Percentage of Growth Inhibition Zone

The observed percentage of the growth inhibition zone of *Phyllosticta zingiberi* for each treatment is presented in Appendices 1–4. Analysis of variance (ANOVA) revealed highly significant differences among treatments. To determine which treatments differed significantly, Duncan's Multiple Range Test (DMRT) was performed, and the results are presented in Table 1.

**Table 1.** Percentage of the growth inhibition zone of *Phyllosticta zingiberi* on PDA medium after data transformation.

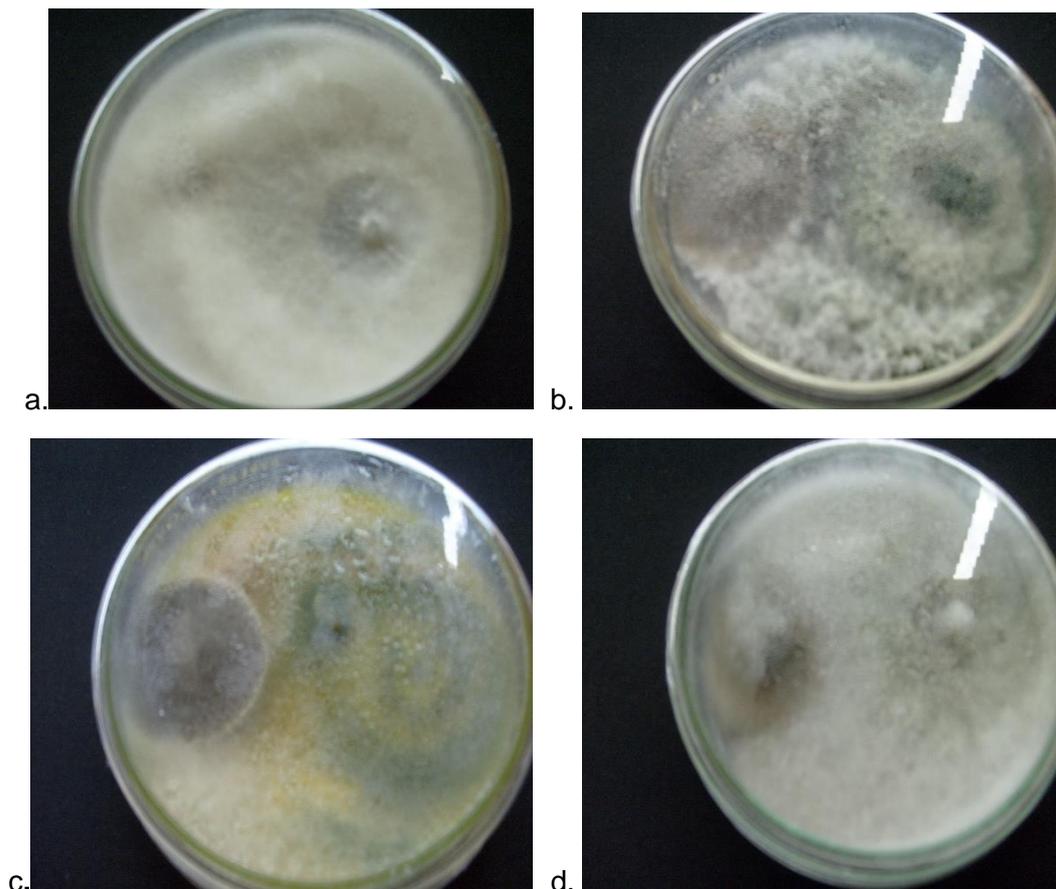
Biological Control Agent (Treatment)	Observation (Day)			
	I	II	III	IV
A0= Control	0.00	12.02d	9.76d	6.43d

K = <i>T koningii</i>	0.00	42.07a	40.86b	41.38b
H= <i>T harzianum</i>	0.00	44.41a	43.59a	46.15a
G= <i>G.spp</i>	0.00	38.28b	45.5a	46.23a
V= <i>G virens</i>	0.00	27.24c	27.24c	27.24c

**Note:** Values in the same column followed by the same letter are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% significance level.

As shown in Table 1, the highest inhibition of *Phyllosticta zingiberi* growth was recorded in treatments with *Gliocladium* spp. (46.23%) and *Trichoderma harzianum* (46.15%), followed by *Trichoderma koningii* (41.38%) and *Gliocladium virens* (27.24%). The lowest inhibition was observed in the control (A0; 6.43%), These results indicate that *Gliocladium* spp. exhibits strong antagonistic activity against *P. zingiberi* (Figure 9a), *Trichoderma harzianum* showed the second-highest inhibitory effect after *Gliocladium* spp. (Figure 9b), indicating that the presence of antagonistic fungi significantly suppressed pathogen growth. The strong antagonistic activity exhibited by *Gliocladium* spp. aligns with recent findings demonstrating that isolates of *Gliocladium* can produce secondary metabolites with antifungal properties, including volatile organic compounds (VOCs) and non-volatile toxins that inhibit pathogenic fungi. For example, recent work by Gupta *et al.* (2021) showed that *Gliocladium* isolates significantly reduced mycelial growth of several phytopathogens through combined mechanisms of antibiosis and mycoparasitism under in vitro conditions. These antifungal compounds disrupt membrane integrity and interfere with cellular metabolism in target pathogens. Similarly, the high inhibitory effect of *T. harzianum* supports its well-documented role as an effective biocontrol agent in numerous pathosystems. Modern studies have highlighted that *T. harzianum* exerts antagonism not only through competition for space and nutrients but also via the production of hydrolytic enzymes (e.g., chitinases and glucanases) and antifungal metabolites that degrade pathogen cell walls. For instance, Li *et al.* (2022) demonstrated that *T. harzianum* consistently reduced the growth of multiple foliar pathogens by secreting enzymes that degrade structural components of fungal cell walls, in addition to inducing host defense responses via systemic acquired resistance pathways.

In addition to enzyme secretion, recent research has emphasized the role of secondary metabolites such as gliotoxin, viridin, and peptaibols in biocontrol activity. These compounds can disrupt cell membranes and inhibit fungal spore germination. For example, Bhattacharyya *et al.* (2023) provided molecular evidence that antagonistic fungi produce metabolites that destabilize pathogen membrane integrity and interfere with signaling pathways in target fungi, resulting in reduced virulence and slower growth. Furthermore, *Trichoderma* spp. are recognized for their ability to trigger induced systemic resistance (ISR) in host plants, enhancing plant defense mechanisms against pathogens. Recent field studies by Wang *et al.* (2024) showed that pre-treatment of crop seedlings with *T. harzianum* markedly decreased disease severity upon pathogen challenge, suggesting that ISR contributes to overall disease suppression beyond direct antagonism. Taken together, these findings confirm that *Gliocladium* spp. and *T. harzianum* possess multiple modes of action that contribute to their superior antagonistic effects against *P. zingiberi*. The observed inhibition patterns are consistent with integrated biocontrol strategies reported in current literature, which emphasize synergistic actions of enzymatic degradation, secondary metabolites, and host resistance induction for effective disease management.



**Figure 9.** In vitro antagonistic activity of biological control agents against *Phyllosticta zingiberi* on PDA medium: (a) Growth inhibition by *Gliocladium* spp.;(b) Growth inhibition by *Trichoderma harzianum*;(c) Growth inhibition by *Trichoderma koningii*; (d) Growth inhibition by *Gliocladium virens*.

In treatments with *Trichoderma koningii* and *Gliocladium virens* (Figures 9c and 9d), an increase in inhibition of *Phyllosticta zingiberi* growth was observed. This enhanced antagonism may be attributed to multiple mechanisms of action exhibited by these fungi. Both *T. koningii* and *G. virens* are known to engage in mycoparasitism, whereby the antagonists attach to and penetrate the hyphae of the pathogenic fungus, leading to degradation of the pathogen's cell walls. Recent analyses of fungal antagonists confirm that mycoparasitism involves direct hyphal contact and enzymatic breakdown of pathogen cell wall components, often mediated by cell wall-degrading enzymes such as cellulases and proteases. (Chen, S., et al, 2024).

In addition to mycoparasitism, *Trichoderma* spp. synthesize a variety of secondary metabolites that exert antifungal effects. These include peptaibols (e.g., *trichokonins*), *gliovirin*, and *viridins*, which have been shown to disrupt pathogen cell integrity and inhibit spore germination and hyphal growth. For instance, antimicrobial compounds produced by *T. koningii* and *T. virens* have demonstrated inhibitory effects against a range of plant pathogens in recent antagonism studies. (Nazia Manzar et al.,2022)

Competition for nutrients and ecological niches also contributes to biocontrol efficacy. *Trichoderma* species are characterized by rapid colonization, efficient nutrient uptake, and production of siderophores that sequester essential micronutrients (iron), thereby depriving pathogens of critical growth resources. Such competitive interactions are increasingly recognized as key components of successful biological control systems.(Suwendu Das and Pil Joo Kim. 2025). Thus, the observed inhibition of *P. zingiberi* by *T. koningii* and *G. virens* likely reflects a combination of parasitic interaction, production

of antifungal metabolites, and competitive exclusion, consistent with contemporary findings in the literature on fungal biocontrol mechanisms.

## CONCLUSION

The study demonstrated that *Gliocladium* spp. and *T. harzianum* are the most effective antagonists against *P. zingiberi*, achieving growth inhibition of 46.23% and 46.15%, respectively. *T. koningii* and *G. virens* showed moderate effects, while the control exhibited minimal inhibition (6.43%). The antagonistic activity is likely mediated by mycoparasitism, antifungal metabolites, and competition for resources. These findings suggest that *Gliocladium* spp. and *Trichoderma* spp. are promising candidates for environmentally friendly management of leaf spot disease in red ginger.

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