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Antagonistic and Antifungal Activities of Endophytic Fungi Isolated from *Fragaria x Ananassa* Against *Colletotrichum Acutatum*

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Abstract

Fragaria x ananassa or strawberry has significant economic value worldwide. However, its cultivation faces various challenges. One example was fungal diseases, such as anthracnose, caused by *colletotrichum acutatum*. Disease management generally relies heavily on synthetic fungicides, raising concerns about environmental sustainability and human health risks. Therefore, more environmentally friendly alternatives are needed, such as the use of biological agents. This study aims to assess the potential of endophytic fungi from strawberry plants as natural biocontrol agents against *colletotrichum acutatum*. The study began with the isolation of endophytic fungi from strawberry tissues and their characterization morphologically. The antagonistic activity of endophytic fungi against *colletotrichum acutatum* was evaluated using three methods: a dual culture assay to assess direct inhibition, a cell-free filtrate assay to analyze the effect of fungal metabolites, and a volatile organic compound (VOC) assay to inhibit the growth of pathogenic pathogens. In this study, 20 fungal isolates were successfully isolated. A total of 15 showed antagonism against *colletotrichum acutatum*. Isolate A1 showed potent inhibition in the dual culture test by effectively antagonizing *colletotrichum acutatum*. Isolate D1 produced extracellular compounds that reduced the growth of *colletotrichum acutatum* by 52.28% ± 3.21% in the cell-free filtrate assay. At the same time, isolate D3 proved most effective in the VOC assay, suppressing the growth of pathogenic colonies by 71.35% ± 3.37%. Thus, endophytic fungi, especially isolates A1, D1, and D3, have the potential to serve as an environmentally friendly alternative to combat anthracnose disease on strawberries.

Keywords: Antagonistic, Antifungal, *Colletotrichum Acutatum*, Endophytic Fungi, Strawberry Plants.

I. INTRODUCTION

Fragaria x ananassa (Strawberries) are among the most economically valuable fruits due to their widespread popularity. Strawberries are not only delicious but also rich in vitamins, antioxidants, and bioactive compounds, which makes them highly sought after. However, growing and preserving strawberries poses significant challenges. Infectious diseases such as those caused by fungi, bacteria, and viruses are one of the most significant impediments to growing strawberries. Anthracnose is a common disease that affects strawberry plants, caused by the pathogenic fungus of the genus *Colletotrichum* [1]. This genus has several species, including *colletotrichum acutatum*, *colletotrichum gloeosporioides*, and *colletotrichum fragariae*, all of which are recognized as damaging the strawberry plants [2]. *colletotrichum acutatum*, a species reported to infect strawberries and a well-known pathogen of carrots, often causes fruit rot [2]. This infection presents small blackish-brown spots on the fruit that may enlarge, eventually causing the fruit to rot and become soft and mushy [3]. Untreated anthracnose can severely damage strawberry crops, affecting both their quantity and quality.

Farmers have used synthetic fungicides to control these outbreaks for decades. They work but using them excessively raises some genuine concerns. An increasing number of consumers are seeking chemical-free strawberries, concerned about the potential health risks associated with these substances. Imazalil (IMZ), chlorothalonil (CTL), and carbendazim (CBZ) are some of the fungicides that have been demonstrated to be toxic to human colorectal epithelial cells (Caco-2), causing apoptosis, oxidative stress, and altering the cell cycle [4]. However, heavy utilization of synthetic fungicides has also been associated with environmental risks such as soil and water contamination [5]. To address such concerns, alternative, sustainable strategies for disease control are being investigated. A practical alternative is the use of endophytic fungi as biological control agents [6]. Furthermore, endophytic fungi are microorganisms that inhabit the healthy tissues of plants without fatigue to the host [7]. These fungi are recorded from various plant organs, including roots, seeds, leaves, flowers, branches, and stems [8]. They are beneficial, mutualistic fungi that are not just residents but also endophytic fungi. Indeed, endophytic fungi could be a future source of diverse bioactive compounds, including anticancer, antiviral, antibacterial, and insecticidal agents, as well as plant growth hormones [9]. For instance, endophytic fungi can also promote strawberry plant growth, with *Trichoderma harzianum* fungi able to produce a 38% increase in total yield, for example, [10], [11].

Endophytic fungi are promising biological control agents that can be utilized to combat plant diseases [12]. Endophytic fungi are highly beneficial and promising, as they aid in restoring plant growth [13]. This enhancement of plant growth results from a mutualistic symbiotic association between fungi and plants, which increases plant tolerance to stress and disease [14]. Despite the potential of utilizing endophytic fungi to control plant diseases, studies have not thoroughly explored their effectiveness against *colletotrichum acutatum* in strawberries. While benefits from endophytic fungi have been reported and utilized in plants to combat other diseases, their role in combating *colletotrichum acutatum* in strawberries has not been thoroughly examined. As an example, *Trichoderma* sp. and *Fusarium* sp. on strawberry fruit, as well as *Mucor* sp. in the strawberry leaves, indicate the presence of beneficial endophytes [15]. However, the antagonistic activity of these isolates against *colletotrichum acutatum* has not yet been tested. Thus, this present study evaluates the antagonistic activity of endophytic fungi isolated from strawberry plants against *colletotrichum acutatum*.

II. METHOD

In this study, researchers investigate the antifungal activity of endophytic fungi isolated from *fragaria x ananassa* (strawberry) against *colletotrichum acutatum*, the causative agent of anthracnose disease. The pathogen poses a significant threat to strawberry production, as it can cause fruit rot, resulting in economic loss to farmers. Due to the harmful effects of chemical fungicides, the present study aimed to screen endophytic fungi for biological control, as they are considered eco-friendly and sustainable sources. The research was structured in five primary steps. The strawberry plant samples were obtained and prepared, and endophytic fungi were isolated and identified. Subsequently, the fungi were identified based on their morphological and physiological features. The fourth stage involves analyzing the antagonistic activity between endophytic fungi and the pathogenic fungus *colletotrichum acutatum*. The cell-free filtrate and volatile organic compound tests were used to evaluate antifungal activity—morphological characterization of different endophytic fungal isolates obtained from the isolation process. To verify the reliability of the results, percentage inhibition data related to antifungal activity were obtained from experiments performed in triplicate. Results are given as averages with standard deviations. Figure 1 describes the outline of the whole research procedure, including stepwise isolation, characterization, and antifungal testing of endophytic fungi against *colletotrichum acutatum*.

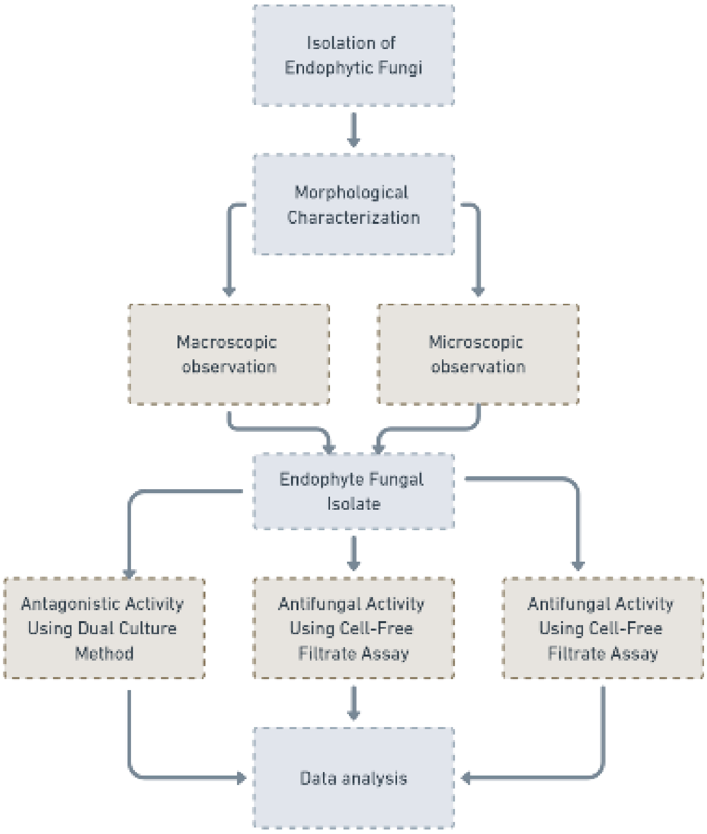

Figure 1 Flowchart of Research Methodology: Isolation, Characterization, and Antifungal Evaluation of Endophytic Fungi Against *Colletotrichum Acutatum*

Healthy strawberries (*fragaria x ananassa*) were obtained from the Strawberry Garden in Banyuroto Village, Sawangan, Magelang, Central Java, for this study. All specimens selected were fresh and without signs of wilting, deformities, or disease. The plants were thoroughly collected and transported to the laboratory for testing. When I arrived at the lab, my first step was to wash the plants under running water. Meanwhile, this procedure was necessary to eliminate dirt, dust, and possible contaminants, which would ensure the reliability of the upcoming experiments. According to the protocols described by [16], the isolation process began with surface sterilization. This important step was performed to isolate only endophytic fungi (those living within the tissues of the plant) and prevent the isolation of their external fungi. These plants were then separated by organ after washing: roots, stems, and leaves. Each was chopped into solid 3 cm long segments. The sterilization process then took place. To disinfect the plant segments thoroughly, they were placed in a solution of 70% (v/v) ethanol for 1 min, allowing the alcohol to wad. Then, they were dipped in 0.5% (v/v) sodium hypochlorite for three minutes to remove any remaining contamination. Then, the segments were re-immersed in 70% (v/v) ethanol for 30 seconds and rinsed twice in sterile distilled water to remove any remaining chemical residues.

After the sterilized segments were laid out in a sterile Petri dish to dry in a sterile environment under conditions conducive to the following steps, portions of the segments (1–2 cm) were cut for experimentation with a sterile surgical scalpel to ensure that no contamination was possible. These samples were subsequently inoculated onto PDA media containing 100 µg/mL of chloramphenicol to promote growth in a controlled environment, thereby restricting the growth of adventitious microorganisms. The organs were coded, and each organ sample was prepared successfully in triplicate. To verify the effectiveness of the surface sterilization, 0.1 mL of sterile distilled water from the final rinse was spread onto PDA media as a negative control using the diffusion method. The organ samples were incubated at room temperature (25-28°C) for 7 days. The fungal isolates that successfully grew were subcultured into PDA media to observe their macroscopic and microscopic characteristics. Furthermore, the characterization of fungal morphology was performed both macroscopically and microscopically. Macroscopic observations were conducted by examining the morphology of the fungal mycelium, including colony color, shape, and surface texture. Microscopic observations were conducted by preparing a pure culture of the endophytic fungus. The isolates were aseptically transferred to an object slide using a sterile inoculating loop. Each preparation was then stained with Lactophenol cotton blue and examined under a light microscope. Observations included the structure of the hyphae (septate or aseptate).

The antagonistic activity of endophytic fungi against *colletotrichum acutatum* was assessed using the dual culture method described by [17]. Pure culture pieces of *colletotrichum acutatum* and each endophytic fungal isolate (approximately 5 mm) were inoculated into a petri dish containing PDA media with a separation of 3 cm (Figure 2a.). As a negative control, a piece of *colletotrichum acutatum* (approximately 5 mm) was placed in the center of a petri dish containing PDA media. The petri dishes were incubated at room temperature (25-28°C) for 5-7 days until the *colletotrichum acutatum* colony in the negative control filled the petri dish. The fungi were observed daily for 5 to 7 days. Antifungal activity of endophytic fungi against *colletotrichum acutatum* using endophytic fungal cell-free filtrate by dilution method was conducted following the procedure described by [18]. Pure culture pieces of endophytic fungi (approximately 5 mm) were inoculated into 10 mL of PDB media. The endophytic fungal cultures were incubated for 10 days at a temperature of 25-28°C. The fungal cultures were then filtered using filter paper. The filtrate was further filtered through a 0.22 µm sterile syringe filter. The obtained cell-free filtrate was mixed with sterile PDA media to achieve a final concentration of 50% (v/v) and then poured into a petri dish. Pure culture pieces of *colletotrichum acutatum* (approximately 5 mm) were inoculated at three points in the petri dish (Figure 2b.). As a negative control, *colletotrichum acutatum* fungi were inoculated into petri dishes containing only PDA media. The petri dishes were incubated for 5-7 days at 25-28°C until the colony of *colletotrichum acutatum* in the negative control filled the petri dish. The radius of the fungal colony was measured from the outer edge to the center, with measurements taken daily for 5 to 7 days.

Antifungal activity of endophytic fungi against *colletotrichum acutatum* through the production of volatile compounds was assessed using the double petri-dish assay, following the procedure outlined by [19]. Pure culture pieces of *colletotrichum acutatum* (approximately 5 mm) were inoculated in the center of a petri dish containing PDA media. Pure culture pieces of endophytic fungi (approximately 5 mm) were inoculated in the center of separate petri dishes containing PDA media. The two petri dishes were then placed facing each other (with *colletotrichum acutatum* on top and endophytic fungi below) after 1 day of incubation (Figure 2c.). As a negative control, petri dishes containing *colletotrichum acutatum* were paired with petri dishes containing only PDA media. The petri dishes were incubated for 5-7 days at 25-28°C until the colony of *colletotrichum acutatum* in the negative control filled the petri dish. The diameter of the fungal colony was measured daily for 5 to 7 days.

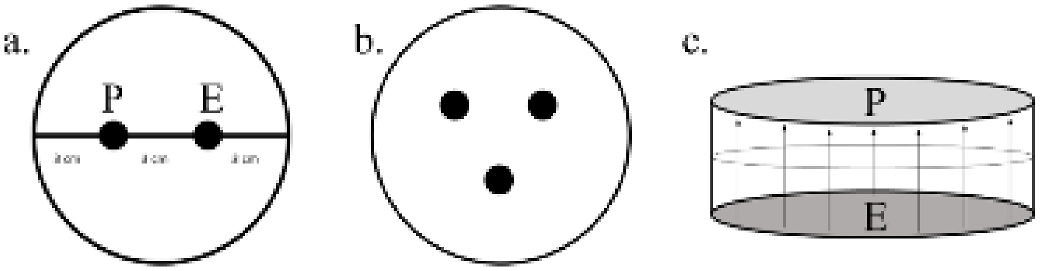

Figure 2 Endophytic Fungi Tests Against C. Acutatum: (a) Dual Culture, (b) Filtrate Assay, (c) Volatile Assay

The percentage of resistance of endophytic fungi to *colletotrichum acutatum* in the antagonism test using the filtrate culture method and inhibition through the production of volatile compounds was calculated based on the formula provided by [20]:

$$PI = \frac{R_1 - R_2}{R_1} \times 100\%$$

Where the percentage of inhibition (PI) was determined in this formula, R1 represented the growth diameter of *colletotrichum acutatum* in the control, and R2 was the growth diameter in the treatment.

III. RESULTS AND DISCUSSION

A. Isolation of Endophytic Fungi from Strawberry Plants (Fragaria x Ananassa)

The isolation of endophytic fungi from various parts of the strawberry plant (*fragaria x ananassa*) revealed a diversity in morphological characteristics and a variation in the number of isolates obtained from each organ (Table 1 and Figure 3). The fungal isolates exhibited differences in colony surface and reverse colors, textures, and hyphal structures. Root isolates (A1 and A2) displayed white and brownish-white colonies with velvety textures, with A1 having septate hyphae and A2 aseptate hyphae. Stem isolates (B1–B6) exhibited diverse colony colors, ranging from white to purplish, yellowish-white, and brownish-white, with most displaying a velvety texture, except for B4 (glabrous) and B5–B6 (cottony). Among them, B1 and B5 exhibited septate hyphae, while the rest were aseptate. Leaf isolates (D1–D6) displayed the most remarkable diversity in colony color, including blackish-white, brownish-white, greenish-white, and purplish-white. Most leaf isolates had cottony textures except for D5 (velvety) and D6 (granular). Almost all of these isolates had aseptate hyphae, except D3 and D6, which were septate. In addition, several isolates from roots stems, and leaves showed the formation of growth zones (+), indicating the expansion of active growth.

Endophytic fungi are microscopic organisms that reside within plant tissues without causing significant damage to their host plants [21]. These fungi can interact with their host plants through mutualistic symbiosis. This mutually beneficial relationship often has a positive impact on plant health. This occurs through mechanisms of increased growth, resistance to pathogens, and tolerance to stress [22]. The isolation of endophytic fungi from various parts of strawberry plants (*fragaria x ananassa*) provides valuable information about the diversity of endophytic fungi that reside within plants. The results of root isolation obtained two isolates (A1, A2). Both isolates exhibited a velvety texture, with one displaying septate hyphae and the other aseptate hyphae. In contrast, the stem is a source of richer fungal diversity, as evidenced by this study, which obtained six different isolates (B1 to B6). Endophytic fungal isolates from the stem exhibited a diverse range of colony colors, including white, yellowish, purplish, and brownish-white, with textures varying from velvety to cottony and a predominance of unbranched hyphae. Similar to the results obtained from the stem, endophytic fungal isolates from the leaves yielded six isolates (D1 to D6), which displayed a mixture of cottony, velvety, and granular textures. Most isolates were unbranched, except for D3 and D6, which were septate.

The results of this study confirm that endophytic fungi can live on a wide variety of plants. Various studies have demonstrated that endophytic fungi can inhabit nearly all plants, exhibiting a high degree of diversity. Additionally, these endophytic fungi are influenced by various factors, including host plant species, specific plant organs, geographic location, seasonal climate, and other environmental conditions [23]. The number of isolates found in various plant organs, such as the stems and leaves of strawberry plants, is generally higher compared to the roots. However, the difference in the number of isolates does not always reflect the full diversity of the endophytic fungal population in each organ. This is because the fungi isolated in this study only represent fungi that can be cultured, while isolates that cannot be cultured may still be present in plant organs.

Table 1 Characteristics of Endophytic Fungi Isolated from Strawberry (*Fragaria* × *Ananassa*)

Organ plant	Isolate	Colony morphology				Hyphae
		Surface Colony	Reverse Colony	Texture	Growing Zone	
Root	A1	White	White	Velvety	-	Septa
	A2	Brownish White	Brownish White	Velvety	+	Asepta
Steem	B1	White	Yellowish White	Velvety	-	Septa
	B2	Brownish White	Purplish White	Velvety	-	Asepta
	B3	Purplish White	Yellowish White	Velvety	-	Asepta
	B4	Yellowish White	Yellowish White	Glabrous	-	Asepta
	B5	White	Yellowish White	Cottony	-	Septa
	B6	White	Brownish White	Cottony	+	Asepta
Leave	D1	Blackish White	Brownish White	Cottony	-	Asepta
	D2	White	Brownish White	Cottony	-	Asepta
	D3	Brownish White	Brownish White	Cottony	-	Septa
	D4	Greenish-White	White	Cottony	+	Asepta
	D5	Purplish White	Yellowish White	Velvety	+	Asepta
	D6	Brownish White	Brownish White	Granular	-	Septa

Table 1 indicates that the endophytic fungi isolated from various parts of the strawberry plant (Fragaria × ananassa) exhibited diverse morphological characteristics. From the root, two isolates (A1 and A2) displayed velvety textures, with A1 being white and septate, while A2 was brownish-white and aseptate, showing growth zone development. The stem yielded six isolates (B1–B6), mostly with velvety textures and color variations ranging from white to purplish and yellowish-white; all were aseptate except B1 and B5, and only B6 showed a growth zone. Among the leaf isolates (D1–D6), colony textures were primarily cottony, with colors varying from blackish-white to purplish and greenish-white. Most leaf isolates were aseptate, except D3 and D6, and three isolates (D4, D5, and D6) exhibited visible growth zones. This variation in morphology, hyphal structure, and growth indicates a high diversity of endophytic fungi inhabiting different strawberry plant organs.

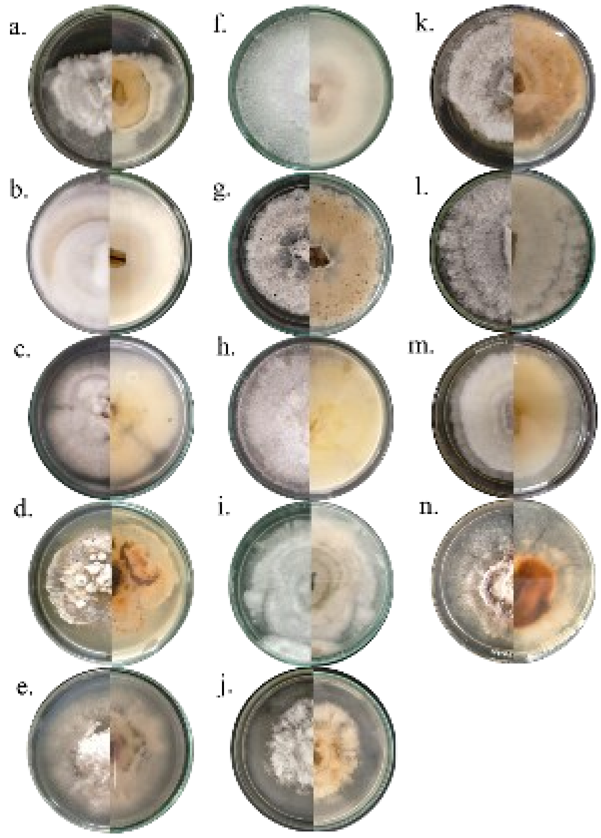

Figure 3 Surface Colony (Left) and Reverse Colony (Right) Morphology of Strawberry Endophytic Fungi

B. Endophytic Fungal Antagonistic Activity Against Colletotrichum Acutatum

Antagonism tests are conducted to evaluate the potential of endophytic fungi as biological control agents. This test was conducted to assess the ability of aggressive fungi to inhibit the growth and activity of phytopathogenic fungi in their presence. Antagonism is typically characterized by two main mechanisms: competition for space and nutrients and antibiosis, which refers to the production of substances that are inhibitory and thus capable of killing or inhibiting the growth of the pathogen. The effectiveness of antagonistic properties is measured by calculating the percentage of inhibition, which indicates the degree to which the growth of pathogenic fungi is suppressed. This percentage serves as a critical indicator of the potential effectiveness of endophytic fungi in biological control applications. The antagonism test employed the dual culture method, comparing endophytic fungi with the pathogen *colletotrichum acutatum*. Observations were made to determine how each fungal isolate affected the growth of the pathogen. The results of the antagonism test revealed varying levels of antagonism among the different isolates (Figure 4). Figure 4 illustrates the growth pattern of *colletotrichum acutatum* and various endophytic fungi on PDA media after seven days of incubation grown on the same petri dish. The control plate (Figure 4a) showed normal growth of *colletotrichum acutatum* without competing fungi, while the presence of endophytic fungi (Figures 4b–4o) resulted in varying levels of inhibition. In each dual culture plate, a piece of endophytic fungal isolate of approximately 3 mm was positioned on the left, while the pathogen *colletotrichum acutatum* was on the right. Some endophytic isolates, such as A1 (Figure 4b), A2 (Figure 4c), and D2 (Figure 4k), showed vigorous antagonistic activity by outcompeting the pathogen and significantly limiting its growth, indicating their potential as effective biological control agents.

In contrast, isolates such as B3 (Figure 4f) and D3 (Figure 4l) showed moderate antagonism, where the growth of the endophyte and pathogen was relatively balanced, indicating intense competition. Meanwhile, isolates such as B6 (Figure 4i) and D6 (Figure 4o) were less effective, with *colletotrichum acutatum* dominating the plate and suppressing the growth of endophytic fungi. Based on the results obtained, the endophytic fungi found in strawberry plants are highly diverse and exhibit varying competitive abilities against pathogenic fungi, thereby demonstrating different potential for biological control. In the limited environment of agar plates, space is a crucial resource for endophytic fungi, and *colletotrichum acutatum* must compete for it. Effective endophytes quickly colonize available surfaces in their environment, which physically restricts the spread of pathogens. These endophytic fungi also absorb essential nutrients from their surroundings, outcompeting pathogens like *colletotrichum acutatum* and limiting their growth. This competition primarily involves the contest for space and nutrients. The ability of endophytic fungi to suppress pathogens often depends on their capacity to outcompete them for space and nutrients [24].

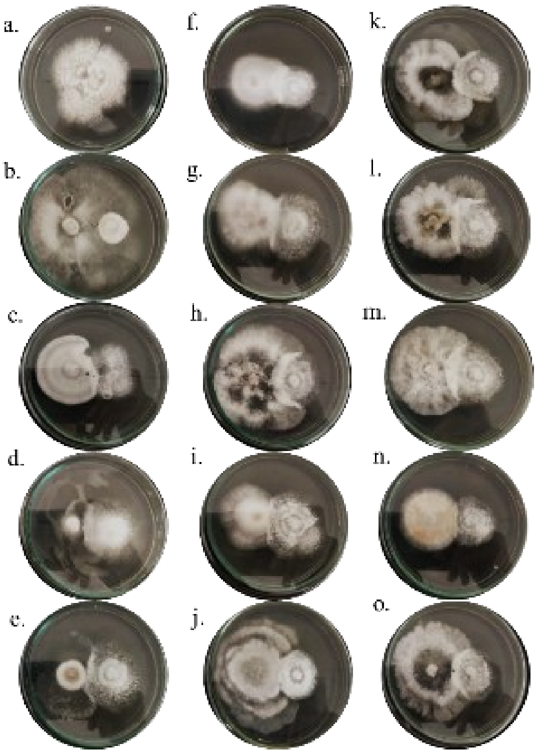

Figure 4 Dual Culture Growth of *Colletotrichum Acutatum* and Fungal Endophytes on PDA Medium

C. Antifungal Activity via Dilution of Cell-Free Filtrate Against Colletotrichum Acutatum

As a result, the endophytic fungal isolates under investigation exhibit antifungal effects, which may be attributed to natural compounds synthesized to suppress the growth of fungal pathogens, such as *colletotrichum acutatum*. Given that endophytic fungi produce a range of secondary metabolites, which have antifungal, antibacterial, and antiviral activity [25]. These compounds are part of a fungus's arsenal of defensive strategies—they help the fungus survive and compete in its environment of plant hosts. To characterize these properties, researchers typically collect a cell-free filtrate, which is obtained by filtering the fungal cells out of their culture medium. This filtrate contains the metabolites that fungi secrete as they develop and is crucial to understanding how these endophytic fungi could play a role in counteracting dangerous pathogens in their natural environments. Understanding these interactions could pave the way for new, natural approaches to managing plant diseases.

The variation in inhibition percentages observed among the different isolates suggests that each strain produces different types or quantities of antifungal compounds (Figure 5 and Table 2). Figure 5 presents the antifungal activity assay of endophytic fungi against *colletotrichum acutatum* using the dilution method with cell-free filtrates from fungal cultures. The control plate (Figure 5a) shows the normal growth of *colletotrichum acutatum* without any treatment, serving as a baseline for comparison. In contrast, Figures 5b–5j illustrate the varying degrees of fungal inhibition observed in the presence of filtrates from different endophytic fungal isolates. Among the tested isolates, D1 (Figure 5g) exhibited the highest inhibition percentage (52.28% ± 3.21%), indicating strong antifungal potential due to the production of bioactive metabolites. Isolate D5 (Figure 5i) also demonstrated substantial inhibition (45.54% ± 2.62%), suggesting its effectiveness in suppressing *colletotrichum acutatum*. Conversely, isolates B4 (Figure 5d) and D6 (Figure 5j) showed the lowest inhibition percentages (33.79% ± 5.79% and 33.83% ± 1.44%, respectively), implying a weaker antifungal effect. Overall, these results demonstrate the potential of secondary metabolites from endophytic fungi as expert antifungal agents, suggesting them as putative natural biocontrol agents. The inhibition of each isolate's differences means that each isolate may produce different types and amounts of antifungal compounds. This finding is consistent with earlier reports showing that various endophytic fungi such as *Aspergillus terreus*, *Armillaria mellea*, and several species of *Xylaria* have the potential to produce potent antifungal metabolites.

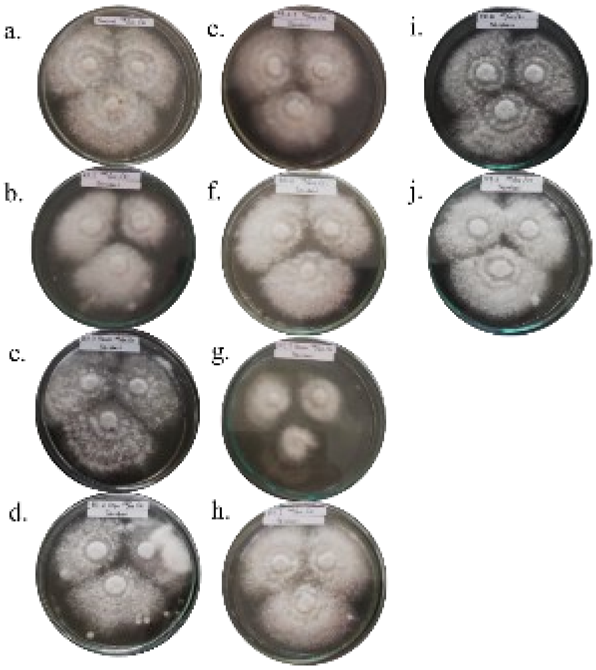

Figure 5 Antifungal Assay Using Cell-Free Filtrate Against *Colletotrichum Acutatum*

In conclusion, isolate D1 was found to be promising for antifungal applications, illustrating that endophytic fungi are a valuable source of natural biopesticides. This finding aligns with previous work showing that these fungi can produce potent antifungal bioactive metabolites. For example, *Aspergillus terreus*, isolated from *Moringa oleifera* leaves, has been proven to exhibit antifungal activity [26]. Fungal endophytes have the potential to synthesize a broad array of antifungal secondary metabolites, such as alkaloids, terpenoids, and polyketides, which could be utilized for biopesticide formulation [27]. Moreover, bioactive compounds from species such as *Armillaria mellea* and the genus *Xylaria* are effective in inhibiting several fungal pathogens [28].

Table 2 Inhibition (%) of *Colletotrichum Acutatum* by Endophytic Fungi via Filtrate and VOC Assays

Isolate	Percentage of Inhibition (%)	
	Fungal Cell-free Filtrate Assay	VOCs Production Assay
A1	41,50 ± 3,45	37,22 ± 8,74
A2	00,00 ± 0,00	00,00 ± 0,00
B1	00,00 ± 0,00	00,00 ± 0,00
B2	00,00 ± 0,00	64,58 ± 6,20
B3	34,59 ± 2,51	47,11 ± 8,10
B4	33,79 ± 5,79	46,62 ± 7,21
B5	37,71 ± 1,90	56,14 ± 3,32
B6	36,08 ± 6,83	64,79 ± 5,90
D1	52,28 ± 3,21	00,00 ± 0,00
D2	00,00 ± 0,00	60,71 ± 0,46
D3	36,10 ± 4,65	71,35 ± 3,37
D4	00,00 ± 0,00	52,90 ± 3,21
D5	45,54 ± 2,62	00,00 ± 0,00
D6	33,83 ± 1,44	43,95 ± 6,12

The inhibition assay results in Table 2 show that several endophytic fungal isolates demonstrated varying degrees of effectiveness against Colletotrichum acutatum through both cell-free filtrate and volatile organic compound (VOC) production. Isolates A1, B3, B4, B5, B6, D1, D3, D5, and D6 exhibited notable inhibition in the filtrate assay, with D1 (52.28%) and D5 (45.54%) showing the highest activity. In contrast, isolates B2, B6, D2, D3, and D4 displayed strong antifungal activity via VOCs, with D3 achieving the highest inhibition at 71.35%. Interestingly, some isolates (A2, B1) showed no inhibitory effect in either assay, while others (e.g., B2 and D2) were inactive in filtrate assays but highly effective in VOC production. These results suggest that endophytic fungi exhibit distinct antifungal mechanisms, with some relying on diffusible metabolites and others on volatile compounds.

D. Antifungal Activity of Endophytic Fungi via Volatile Compound Production

Fungal endophytes have been recognized to produce a variety of volatile organic compounds (VOCs), which have emerged as potentially potent antifungal agents for biocontrol applications [29]. They are tiny, lightweight chemical substances capable of dispersing through the air and coming into contact with other organisms without physical contact [30]. The difference in percentages of inhibition in antifungal activity tests indicates the differing efficacy of these compounds among different fungal isolates. This variability implies that some isolates generate more potent VOCs than others. Figure 6 illustrates the antifungal activity of endophytic fungi against *colletotrichum acutatum* through the production of volatile organic compounds (VOCs) using a double petri-dish assay. In this setup, the growth of *colletotrichum acutatum* (top plate) and the fungal endophyte (bottom plate) on the PDA medium were observed after seven days of incubation.

The control plate (Figure 6a) represents the normal growth of *colletotrichum acutatum* in the absence of VOC exposure, serving as a baseline for comparison. Among the tested isolates, B2, B6, and D3 demonstrated the highest levels of inhibition, with inhibition percentages of 64.58% ± 6.20%, 64.79% ± 5.90%, and 71.35% ± 3.37%, respectively. The strong antifungal effects of these isolates suggest that they produce VOCs in high concentrations or that their VOCs are particularly effective in suppressing the growth of *colletotrichum acutatum*. Moderate inhibition was observed in isolates such as A1, B3, B4, B5, D2, D4, and D6, with inhibition percentages ranging from 37.22% ± 8.74% (A1) to 56.14% ± 3.32% (B5). Some isolates produce volatile organic compounds (VOCs) in lesser quantities or ineffective amounts compared to more potent isolates. For instance, A2, B1, and D1 isolates were unable to reduce the inhibition, implying that they either cannot emit VOCs or the emitted VOCs are ineffectual against *colletotrichum acutatum*. This moderate inhibition could be the result of these isolates producing VOCs that are either less potent or at lower concentrations than those of the more effective isolates. Alternatively, these isolates may be generating distinct profiles of VOCs having particular antifungal efficacy, resulting in only partial activity towards *colletotrichum acutatum*. A2, B1, and D1 exhibited no inhibitory action. The unimpeded behavior of these isolates implies either non-accumulation of VOCs or production of VOCs ineffective against *colletotrichum acutatum*.

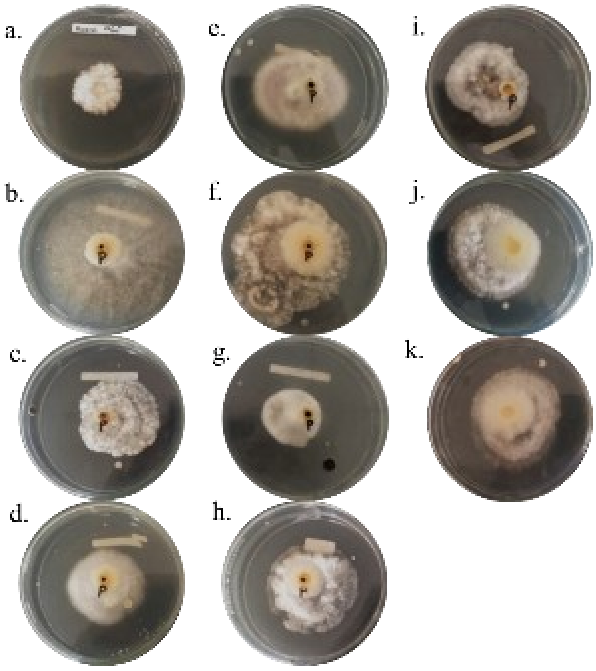

Figure 6 VOC-based Antifungal Assay Against *Colletotrichum Acutatum* Using Double Petri Dishes

Endophytic fungi emit volatile organic compounds (VOCs), which are proving to be a valuable asset in the fight against fungal pathogens [31]. These compounds are promising candidates for antifungal use, especially in sustainable agricultural systems, as they are effective against a wide range of pathogenic fungi. For example, they can target notorious culprits such as *colletotrichum acutatum*, which causes anthracnose in crops like strawberries. The way volatile organic compounds (VOCs) function is fascinating and warrants further attention. They can damage the cell walls and membranes of these phytopathogens, resulting in significant changes to their structure and function. Ultimately, this disruption can cause the cells to leak their contents, which prevents them from surviving and reproducing. Moreover, VOCs are known to alter redox balance, increase intracellular ROS production, and promote membrane lipid peroxidation, mitochondrial dysfunction, and ATP depletion, all of which contribute to their antifungal effects [32]. *Trichoderma asperellum* and *Trichoderma harzianum* are ascomycete species previously documented to elicit a priming effect in plants due to the exuded secretion of certain VOCs, activating pathogen response pathway-related genes [33], [34]. Cyclohexanol is a previously reported VOC product of *Trichoderma* that can induce antifungal activity in some plant species. At the same time, in the beneficial fungus *Trichoderma atroviride*, the Lox1 gene is required for the production of the plant growth-promoting VOC pentyl-α-pyrone (6-PP) [35]. More importantly, almost all of them are species- or strain-specific, which leads to diverse and effective antifungal activities of the released volatile organic compounds (VOCs) [36]. Several different VOCs have been identified as being produced by fungal endophytes, such as alcohols, aldehydes, furans, ketones, terpenes, esters, acids, and lipids, all of which play a role in the antifungal effect observed [32], [37]. These VOCs are highly specific and diverse, reinforcing the potential of these molecules as targeted, natural biocontrol agents for controlling plant diseases.

IV. CONCLUSION

The study successfully isolated and identified endophytic fungi from different parts of the strawberry plant, highlighting their diversity and potential as biological control agents against *colletotrichum acutatum*. Some of these fungal isolates demonstrated substantial antifungal activity, either by competing with the pathogen and thereby inhibiting its growth or through antibiosis or the production of volatile compounds that interfere with the pathogen's cellular functions. These results suggest that endophytic fungi, particularly those with high antagonistic activity, can serve as natural alternatives to synthetic fungicides in combating strawberry anthracnose. Further studies are needed to gain a deeper understanding of the molecular mechanisms governing these interactions and to explore the application of fungi in field conditions to assess efficacy and safety under actual agricultural settings.

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