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Endophytic microbes from red betel suppress *Colletotrichum acutatum* causing anthracnose in chili pepper

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Abstract

Anthracnose is a major disease that reduces chili production by 50–100%, leading to significant economic losses. The use of synthetic fungicides remains common; however, their application is limited due to environmental concerns and residual effects on post-harvest products. Therefore, the development of environmentally friendly biological control methods is critically important. This study aimed to evaluate the potential of endophytic microbes isolated from *Piper crocatum* leaves as biocontrol agents against anthracnose caused by *Colletotrichum acutatum* in chili plants. The methodology involved isolating endophytic microbes from *Piper crocatum* leaves, conducting *in vitro* antagonism tests via volatile inhibition assays, and performing morphological and biochemical analyses to elucidate the mechanisms of antagonism. Results indicated that isolate SM2 showed the greatest potential to suppress *C. acutatum* growth, achieving a maximum inhibition rate of 53% in the volatile test. These findings suggest that endophytic microbes from *Piper crocatum* leaves have promising potential as biological control agents for managing anthracnose in chili, thereby reducing reliance on excessive chemical fungicide applications.

Keywords: endophytic microbes; *Colletotrichum acutatum*; biological control

1. Introduction

Capsicum annuum L., commonly known as chili pepper, is a vital horticultural crop with significant economic importance in Indonesia, serving both domestic markets and industrial sectors. The sustained high demand and stable market conditions have driven continuous cultivation efforts. In 2023, chili pepper consumption reached approximately 2.19 kg per capita per year, with projections indicating a continued increase through 2026. However, production fluctuations have been observed, declining from 1,544,441 tons in 2022 to 1,506,762 tons in 2023, and subsequently rising again to around 1.56 million tons in 2024. These fluctuations highlight underlying cultivation challenges, notably disease pressures such as anthracnose caused by *Colletotrichum* spp., which can severely reduce both yield quality and quantity, resulting in substantial economic losses (Luis et al., 2025). Anthracnose manifests as black lesions on the fruit surface, which can develop into soft rot, leading to desiccation, wilting, and premature fruit drop. The disease affects both pre- and post-harvest stages and can persist during storage, exacerbating post-harvest losses. Currently, disease management primarily relies on chemical fungicides containing beno-

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myl. While these agents are effective in the short term, excessive reliance can lead to pathogen resistance, residual chemical contamination, and ecological imbalance.

In response, there is a growing emphasis on sustainable and eco-friendly disease control strategies. Among these, the use of endophytic microbes as biological control agents has garnered considerable attention. Endophytic yeasts are microorganisms that can reside asymptomatically within plant tissues and promote plant health through multiple mechanisms. These microbes can enhance plant growth by synthesizing phytohormones such as indole-3-acetic acid (IAA), increasing nutrient availability, and stimulating root development and biomass accumulation. Additionally, endophytic yeasts exhibit biocontrol activity through mechanisms such as competition for space and nutrients, the production of hydrolytic enzymes, the secretion of antimicrobial compounds, and the induction of systemic resistance in host plants. Recent research has demonstrated that endophytes isolated from *Capsicum annuum* itself possess considerable potential as biocontrol agents against *Colletotrichum* spp. and other phytopathogens. These endophytic yeasts have shown antagonistic activity, suggesting their utility as sustainable, natural alternatives to chemical fungicides. This approach aligns with principles of environmentally sustainable agriculture and offers a promising strategy to reduce chemical inputs while enhancing disease resistance in chili crops. Therefore, investigating the diversity and efficacy of endophytic yeasts derived from *Capsicum annuum* as biocontrol agents against anthracnose disease represents a vital step toward developing integrated disease management strategies that are effective, sustainable, and environmentally friendly.

2. Materials and Methods

This study used red betel (*Piper crocatum*) leaf samples, red chili pepper seeds var. Tanjung 2, endophytic microbial isolates obtained from exploration, *Colletotrichum acutatum* inoculum, Potato Dextrose Agar (PDA), alcohol 70%, sodium hypochlorite (NaOCl) 3%, sterile distilled water, NPK fertilizer, propineb fungicide, planting media, and polybags. The equipment used included Petri dishes, Erlenmeyer flasks, test tubes, micropipettes, microscopes, incubators, autoclaves, laminar airflow cabinets, sprayers, and other laboratory tools. The experiment was arranged in a completely randomized design (CRD) with six treatments: P0 = control; P1 = isolate SM1; P2 = isolate SM2; P3 = isolate SM3; P4 = isolate SM4; and P5 = propineb fungicide, with four replications. Each experimental unit consisted of 5 chili fruits, for a total of 120. The observed parameters included incubation period, disease incidence, disease severity, AUDPC (Area Under Disease Progress Curve), infection rate, plant height, and number of fruits.

2.1. Isolation of yeasts from plants of the family Piperaceae

The endophytic yeast isolates used in this study were obtained from a pre-existing collection. These isolates were previously isolated from healthy plant tissues of the family *Piperaceae* through exploration and isolation efforts conducted by a private collection. Therefore, this study did not perform initial isolation procedures but instead utilized well-characterized isolates for subsequent testing aligned with the research objectives.

2.2. Preparation of Colletotrichum acutatum inoculum

The pathogenic *C. acutatum* isolate used in this study was obtained from a private collection. The inoculum was prepared by subculturing the isolate onto fresh Potato Dextrose Agar (PDA) plates using sterile inoculation loops, and incubating at room temperature in the dark for 7 days. Isolate identification was performed macroscopically and microscopically prior to inoculum preparation. To obtain the spore suspension, colonies were misted with sterile distilled water and gently agitated for approximately one minute to dislodge spores from the colony surface. The resulting suspension was harvested using

a sterile inoculation loop and filtered through Whatman filter paper to remove mycelial fragments and hyphae. Spore concentration was subsequently determined using a hemocytometer, and the suspension was diluted with sterile distilled water to a final concentration of 10^6 conidia/mL.

2.3. Pathogenicity test of Colletotrichum acutatum

The pathogenicity test was conducted to confirm that the fungal isolate is responsible for anthracnose disease. Surface-sterilized chili fruits were aseptically wounded and then inoculated with 0.1 mL of the spore suspension. The suspension of *Colletotrichum acutatum* was prepared at a concentration of 10^5 conidia/mL. Subsequently, the fruits were incubated under humid conditions at room temperature and observed over 7 days to determine the incubation period and symptom development.

2.4. Mass cultivation of endophytic yeasts

Mass cultivation of the endophytic yeasts was carried out by preparing an initial inoculum. Preparation began by sterilizing Potato Dextrose Agar (PDA) medium in an autoclave at 121°C for 15–20 minutes. Endophytic yeast isolates from plants of the family *Piperaceae*, obtained from a private collection, were sampled using sterile inoculation loops or spatulas. A portion of the colony was inoculated onto PDA medium. The plates were incubated at 28–30°C for 24–48 hours in a closed environment to prevent contamination. The growth of yeast colonies was observed macroscopically based on their characteristic morphology.

Once the yeast colonies had developed, they were rinsed with sterile distilled water, and the cells were gently scraped using a sterile spatula. The cell suspension was then prepared in sterile distilled water in a 50 mL Erlenmeyer flask. The suspension was homogenized thoroughly, and cell density was determined using a hemocytometer. If the concentration was too high, the suspension was diluted to achieve the desired cell density.

2.5. Antagonistic testing of endophytic yeasts against Colletotrichum acutatum

The antagonistic activity of the endophytic yeasts against *C. acutatum* was evaluated using dual culture and volatile compound assays. The percentage inhibition was calculated by measuring the radius of *C. acutatum* growth until day 7 post-inoculation of the yeast. The formula used to determine the inhibition of *Collectotrichum acutatum* mycelial growth by endophytic yeast.

$$\mathrm{I} = \frac{\mathrm{R1} - \mathrm{R2}}{\mathrm{R1}} x\ 100\%$$

Where:

I = Inhibition percentage (%).
R1 = Radius of *C. acutatum* colony growing away from the endophytic yeast colony.
R2 = Radius of *C. acutatum* colony growing toward the endophytic yeast colony.

2.6. Volatile organic compound (VOC) Test

In a Petri dish, the lower plate containing PDA media is inoculated with a 5 mm diameter piece of *C. acutatum*, while the upper plate containing PDA is inoculated with the same diameter of endophytic yeast. After an initial incubation of 2 days, the two plates are stacked and sealed with plastic wrap to prevent gas exchange. The control treatment involves stacking *C. acutatum* with PDA without yeast. All treatments are then incubated for 7 days at room temperature, and observations are made by measuring the diameter of *C. acutatum* colonies on the bottom plate.

$$I = \frac{Dk - Dp}{Dk} x\ 100\%$$

Where:
THR = Relative inhibition rate (%).
Dk = Colony diameter of *C. acutatum* in the control.
Dp = Colony diameter of *C. acutatum* in the treatment.

3. Results

A total of 4 bacterial isolates (SM 1, SM 2, SM 3, and SM 4) were identified based on colony morphological characteristics, including shape, elevation, margin, and color (Table 1). SM 1 exhibited an irregular colony shape with a raised elevation, an entire margin, and a yellow pigmentation. SM 2 also displayed an irregular shape; however, it differed from SM 1 in terms of elevation and margin, showing a convex elevation and an undulate margin, with a yellow colony color. In contrast, SM 3 and SM 4 both formed circular colonies with convex elevation and entire margins. The two isolates were distinguished by colony color: SM 3 produced a pink-pigmented colony, while SM 4 produced a yellow-pigmented colony. Overall, the majority of isolates (SM 1, SM 2, and SM 4) had yellow colonies, while variations in colony shape, elevation, and margin were observed among the isolates.

Table 1. Characteristics of the morphology of the endophytic bacterial colony on a Red Betel.

Isolate code	Colony shape	Elevation	Margin
SM 1	Irreguler	Raised	Entire
SM 2	Irreguler	Convex	Undulate
SM 3	Sferis	Convex	Entire
SM 4	Sferis	Convex	Entire

The hemolytic activity and hypersensitivity reaction of four bacterial isolates (SM 1, SM 2, SM 3, and SM 4) were evaluated using blood agar medium and tobacco plant testing, respectively (Table 2). The results demonstrated that none of the isolates exhibited hemolytic activity, as all four (SM 1, SM 2, SM 3, and SM 4) produced negative results on blood agar, indicating the absence of red blood cell lysis. Similarly, all isolates showed no hypersensitivity reactions when tested on tobacco plants, suggesting that none triggered a hypersensitive response in the host plant. These findings indicate that all four isolates are non-hemolytic and do not elicit a hypersensitivity reaction, which are important safety indicators suggesting their potential non-pathogenic nature toward both animal blood cells and plant hosts.

Table 2. Hemolysis assay of diverse yeast isolates on blood agar medium and hypersensitivity reaction testing in tobacco.

Isolate code	Hemolysis	Hypersensitivy
SM 1	-	-
SM 2	-	-
SM 3	-	-
SM 4	-	-

The antagonistic activity of four bacterial isolates (SM 1, SM 2, SM 3, and SM 4) was evaluated on Potato Dextrose Agar (PDA) medium through two methods: direct antagonistic inhibition and volatile compound inhibition (Table 3). The results revealed that all isolates demonstrated inhibitory activity against the tested pathogen, with varying inhi-

bition rates between the two methods. In terms of direct antagonistic inhibition, SM 2 showed the highest inhibition rate at 45%, followed by SM 3 at 39%, SM 4 at 34%, and SM 1 at 26%. Regarding volatile compound inhibition, SM 2 again exhibited the highest inhibition rate at 53%, followed by SM 3 at 43%, SM 4 at 41%, and SM 1 at 42%. Notably, volatile compound inhibition rates were consistently higher than direct antagonistic inhibition rates across all isolates, suggesting that volatile compounds produced by these isolates may play a more effective role in suppressing pathogen growth. Among all isolates, SM 2 demonstrated the most promising antagonistic potential through both methods, making it the most effective candidate as a biocontrol agent.

Table 3. Antagonistic activity assay on potato dextrose agar (PDA)

Isolate code	Antagonistic inhibition rate (%)	Volatile compound inhibition rate (%)
SM 1	26 %	42 %
SM 2	45 %	53 %
SM 3	39 %	43 %
SM 4	34 %	41 %

4. Discussion

The endophytic microbes isolated from red betel (*Piper crocatum*) demonstrated considerable potential as biological control agents against anthracnose disease caused by *Colletotrichum acutatum* on chili plants. The isolates exhibited diverse colony morphological characteristics on the growth medium, including irregular and circular colony shapes, raised and convex elevations, and entire and undulate margins (Table 1). These morphological variations among isolates are commonly observed in endophytic microbial communities. They may reflect differences in physiological traits and adaptation strategies. However, the precise biochemical basis, such as pigment content or metabolic activity, was not directly measured in this study and warrants further investigation (Suloi et al., 2022).

Preliminary biosafety screening was conducted through hemolysis and hypersensitivity assays to evaluate the safety profile of the isolates prior to further application. All four isolates (SM 1, SM 2, SM 3, and SM 4) yielded negative results in both tests, indicating the absence of hemolytic activity and hypersensitive reaction under the tested conditions. Negative hemolytic activity indicated that the isolates did not produce hemolysins capable of lysing red blood cells, an important preliminary indicator of safety toward mammalian cells (Utami et al., 2025; Utarti et al., 2024). It should be noted, however, that these results represent only a preliminary biosafety screening and do not constitute a comprehensive safety assessment; further toxicological evaluations would be necessary before field-scale application. In the hypersensitivity test conducted on tobacco leaves, no necrotic symptoms were observed in any of the isolates, indicating a non-pathogenic nature toward plant hosts. This is consistent with the characterization of hypersensitive reactions as necrotic symptoms triggered by pathogenic microbial infection and suggests that the isolates are suitable candidates for further evaluation against anthracnose disease in chili plants.

The antagonistic potential of the isolates was assessed using dual-culture and volatile compound assays on Potato Dextrose Agar (PDA) medium (Table 2). In the dual culture method, isolate SM 2 showed the highest inhibition rate (45%), followed by SM 3 (39%), SM 4 (34%), and SM 1 (26%). In the volatile compound assay, SM 2 again demonstrated the highest inhibitory activity at 53% on the seventh day of observation, while SM 1, SM 3, and SM 4 recorded inhibition rates of 42%, 43%, and 41%, respectively. Notably, volatile compound inhibition rates were consistently higher than direct antagonistic inhibition rates across all isolates, suggesting that diffusible volatile metabolites may contribute sub-

stantially to pathogen suppression. The observed inhibitory activity is consistent with previous reports showing that endophytic microbes suppress pathogen growth through competition for nutrients and space in the growth medium (Ropalia, 2017). The production of antifungal volatile compounds has also been documented as an important indirect mechanism, with volatile metabolites reported to interfere with fungal development in spatially separated co-culture systems. It should be emphasized, however, that the precise mechanisms underlying these inhibitory effects, including nutrient competition dynamics and volatile compound characterization, were not fully elucidated in the present study and require further mechanistic investigation.

Among all isolates, SM 2 consistently exhibited the highest antagonistic performance in both assay methods, suggesting a superior capacity to suppress *C. acutatum* growth *in vitro*. The superior performance of SM 2 may be attributed to its greater capacity to produce inhibitory metabolites or its competitive colonization traits. However, these mechanisms were not directly characterized in this study and represent an important direction for future research. Comparable findings have been reported in studies on endophytic microbes from other plant sources, in which select isolates demonstrated significantly higher antagonistic activity, attributed to the production of antifungal secondary metabolites. In greenhouse experiments, endophytic microbial treatments, particularly SM 2, effectively delayed the incubation period and reduced disease incidence, disease severity, AUDPC values, and infection rates compared to the untreated control. These results align with previous studies reporting the efficacy of endophytic microbes in reducing foliar fungal diseases under controlled conditions. Furthermore, endophytic microbial treatments were associated with improvements in plant growth parameters and fruit production, indicating potential growth-promoting effects. However, this relationship should be interpreted cautiously, as the mechanisms linking microbial application to plant growth promotion were not fully characterized in the present study, and confounding factors in the greenhouse environment cannot be entirely excluded. These findings highlight the promise of endophytic microbes isolated from *Piper crocatum*, particularly isolate SM 2, as environmentally friendly biological control agents for managing anthracnose disease in chili cultivation. Future studies should focus on molecular identification of the active isolates, characterization of their bioactive metabolites, and evaluation of their efficacy and safety under field conditions to substantiate their potential for practical application.

5. Conclusions

Endophytic microbes isolated from red betel (*Piper crocatum*) showed potential as biological control agents against anthracnose disease caused by *Colletotrichum acutatum* on chili plants. All isolates passed preliminary biosafety screening, showing negative results in hemolysis and hypersensitivity tests. In vitro assays confirmed that the isolates inhibited pathogen growth through direct antagonism and volatile compound production. Greenhouse experiments showed that microbial treatments reduced disease incidence, severity, and infection rates while improving plant growth and fruit production. Among all isolates, SM 2 demonstrated the best overall performance in both laboratory and greenhouse conditions. These results indicate that endophytic microbes, particularly SM 2, are promising candidates as environmentally friendly biocontrol agents for the management of anthracnose in chili cultivation. Further studies on molecular identification, metabolite characterization, and field trials are needed to confirm their practical applicability.

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