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The Potential of Endophytic Bacteria in Controlling *Cylindrocladium* sp., The Cause of Leaf Blight on *Eucalyptus pellita* Seedlings by *In-Vitro*

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ABSTRACT

Cylindrocladium sp is a fungus that causes leaf blight on Eucalyptus pellita seedlings. Endophytic bacteria are an alternative biological control that can reduce the risk of environmental pollution and reduce resistance by pathogens. This research aims to obtain endophytic bacteria that have potential as biocontrol agents and to characterize isolates that inhibit more than 30%. Testing was carried out using the dual culture method on Potato Sucrose Agar (PSA) media. The results of the antagonism test showed that the endophytic bacterial isolates E320 and E323 were able to form an inhibition zone of more than 30%, namely 38.79% and 41.25% and had the potential to act as biocontrol agents for Cylindrocladium sp., which causes leaf blight on eucalyptus. The characteristics of isolate E320 were that it was able to produce protease, cellulase and siderophore activities, while isolate E323 was only able to produce protease.

Keywords: *Antagonis Bacteria, Endophytic, Eucalyptus, Leaf Blight, and Hidrolytic Enzym*

1. INTRODUCTION

Industrial Forest Plantation (HTI) involves the cultivation of forestry plants using intensive silviculture techniques to cater to the needs of the forest product industry, particularly for pulp and paper production. Eucalyptus pellita is a species commonly grown by companies engaged in industrial forest plantation. The success of an HTI company in boosting roundwood production hinges on the availability of high-quality, healthy, and robust seeds. Cylindrocladium sp. is a known culprit behind leaf blight disease in E. pellita seedlings. Symptoms of Cylindrocladium infection in Eucalyptus plants manifest as leaf spots and shoot blight, spreading from the tree's base upwards. In conditions of high humidity and frequent rainfall, lesions can engulf the entire leaf surface, while infection of young shoot tips can lead to late blight (Chen et al., 2011). In India, Cylindrocladium sp. infection can result in seedling mortality and substantial economic losses (Arya et al., 2011).

Several control strategies have been implemented, such as regulating seed spacing, sanitizing nurseries, utilizing resistant clones, eradication, employing biological agents, and using synthetic fungicides. Trichoderma sp, a biological agent from the fungus group, has been found to reduce the growth of Cylindrocladium sp mycelium by 43.5% (Siregar et al. 2022) and Gliocladium by 19.3% (Amalia et al. 2008). Despite this, these agents have not demonstrated optimal efficacy in the field, leading to the continued consideration of synthetic fungicides. Prolonged use of synthetic fungicides may result in environmental contamination and the development of resistant pathogen populations (Anugrah and Fitri, 2018).

Endophytic bacteria are microorganisms that establish a symbiotic relationship with plants, residing and forming colonies within their tissues without causing any detrimental effects. The composition of endophytic bacteria in a given environment is influenced by factors such as the specific host plant, prevailing environmental conditions, and the characteristics of the bacteria themselves. Certain endophytic bacteria exhibit a broad range of host compatibility and possess the ability to produce various beneficial compounds, including antibiotics and hydrolytic enzymes. These compounds serve to safeguard plants against pathogenic attacks and indirectly enhance their overall health (Afzal et al., 2019). Notably, research conducted by Andriani and Firdaus (2019) demonstrated that endophytic bacteria derived from the paitan plant effectively suppress the Fusarium oxysporum pathogen through the production of antibiosis compounds, consequently promoting plant growth. Additionally, Widiantini et al. (2017) reported findings indicating that endophytic bacteria obtained from rice plants exhibit the capacity to inhibit over 50% of the mycelium of Pyricularia oryzae, the causal agent of rice leaf blast disease, thereby reducing the severity of the disease by 23.90% (Marwan et al., 2021).

According to the aforementioned description, it is necessary to conduct research in order to acquire endophytic bacteria from eucalyptus plants. These bacteria possess the potential to act as biocontrol agents by inhibiting the growth of the fungal pathogen Cylindrocladium sp., which is responsible for causing leaf blight in E. pellita plants.

2. MATERIAL AND METHODS

This research was carried out at the Plant Protection Department and Green House Research and Development Laboratory of Sinarmas Forestry, Perawang, Siak Regency (0°41'51.1"N 101°36'06.5" E). This research starts from April 2022 to May 2022.

The research utilized endophytic bacterial isolates from eucalyptus (PT. Arara Abadi R&D collection) as well as various materials including Nutrient Agar (NA), Chloramphenichole, King's B, skim milk agar (SMA) medium, Fe-CAS medium, NB+L Tryptophan medium, chitin medium, Carboxymethyl cellulose agar (CMC) medium, hydrogen cyanide (HCN) medium, Pikovskya's medium, glycine, Salkowski reagent, Aquades, 70% alcohol, and 96% alcohol. Additionally, the tools employed in the study encompassed petri dishes, test tubes, tubes, bunsens, syringes, filter paper, compound microscopes, erlenmeyers, spatulas, analytical scales, micropipettes, incubator shakers, spectrophotometers, cuvettes, tube needles, and other supporting instruments.

Research Implementation

The four endophytic bacterial isolates utilized in this study were sourced from the R&D collection of PT. Arara Abadi and originated from eucalyptus plants that had not previously undergone testing for disease-causing fungal pathogens. Additionally, the Cylindrocladium sp. pathogen isolate employed in the research was also obtained from the R&D collection of PT. Arara Abadi and underwent initial pathogenicity testing prior to being subjected to in vitro antagonism assays with the endophytic bacteria. Subsequent to the antagonism tests, the obtained results were subjected to analysis using ANOVA, followed by the application of the Duncan Multiple Range Test (DMRT) at a significance level of 5%. The various stages of the research conducted are delineated in the accompanying flowchart:

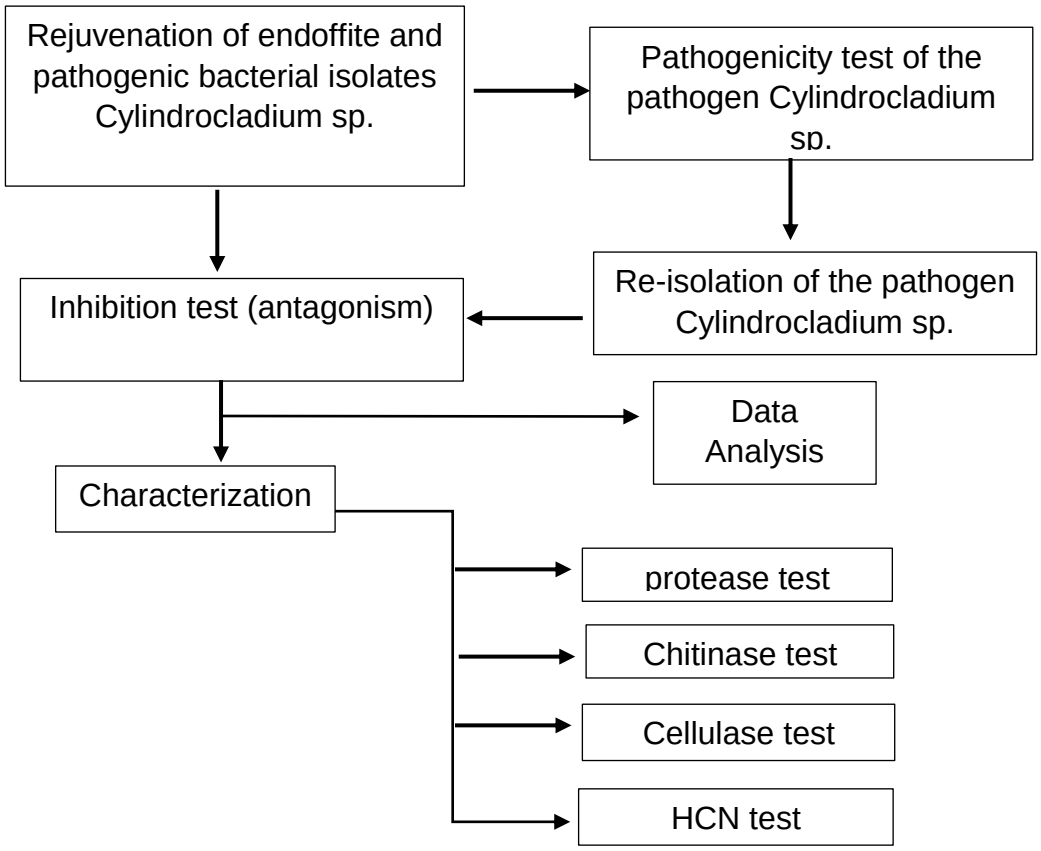

Figure 1. Research Flow Diagram

Rejuvenation of Endoffite and pathogenic bacterial isolates Cylindrocladium sp.

The rejuvenation of endophytic bacterial isolates (E309, E317, E320, and E323) obtained from PT. Arara Abadi's collection was carried out on NA media. These four isolates were subjected to an antagonism test to evaluate their potential in inhibiting pathogenic fungi. The pathogenicity test yielded Cylindrocladium sp., which was cultured on PSA media for a duration of 7 days.

Test of the inhibitory power of endophytic bacteria against Cylindrocladium sp.

Four different strains (E309, E320, E323, and E327) obtained from PT. Arara Abadi were examined for their inhibitory effects against the Cylindrocladium sp fungus through the dual culture technique. The endophytic bacterial strain was streaked on one side of the medium, positioned 3 cm away from the edge of the petri dish containing PSA medium. Subsequently, a 0.5 cm segment of a 7-day-old Cylindrocladium sp culture was placed on the opposite side of the dish. The setup was then left to incubate at room temperature, resulting in the formation of an inhibition zone. This experiment followed a Completely Randomized Design and was replicated thrice. On the 6th day, measurements were taken, and the percentage of inhibition was determined using the following formula:

$$\text{IZ} = \frac{R1-R2}{R1} x100$$

IZ= Bland zone (%), R1= Distance of Cylindrocladium sp mycelium radius towards the edge of the petri dish (cm), R2= Distance of Cylindrocladium sp mycelium radius to the test bacteria (cm).

According to Mori et al. (1997), The ability of antifungal bacteria classifications are as follows: 0% indicates no antifungal activity, 0-25% signifies weak activity, 25.1-50% represents moderate activity, 50-75% indicates strong activity, and > 75% denotes very strong activity.

Protease Test

The qualitative assessment of this test involves the placement of a 0.5 cm paper disc, which has been saturated with a suspension of endophytic bacterial isolates, onto a 1% skim milk agar (SMA) medium. Subsequently, the medium is incubated at room temperature for a period of 24 to 72 hours. The presence of a distinct transparent area signifies the occurrence of proteolytic activity.

Chitinase test

This test was carried out qualitatively by spotting the test isolate on 0.3% chitin agar medium and incubating at room temperature for 4 days. A positive reaction is indicated by the formation of a clear zone around the colony.

Cellulase test

The qualitative test was conducted by streaking the test bacteria on 1% CMCA media (Carboxymethylcellulose Agar) and allowing it to incubate for 48 hours. Subsequently, the petri dish containing the incubated test bacterial isolate was supplemented with 0.1% Congo Red solution and gently shaken. After a 24-hour period, the sample was washed with 1M NaCl. The appearance of a clear zone on the scratch marks of the test bacteria signifies a positive reaction.

HCN generator test

The test was performed qualitatively by cultivating the test bacteria in Glysine media (NA + 4% glycine). A piece of Whatman filter paper no. 1, soaked in CDS (Cyaride Detection Solution) solution, was attached to the lid of the petri dish. Following this, the samples were incubated for a period of 4 days. A change in the color of the filter paper from yellow to light brown to brick red indicates a positive reaction.

Data Analysis

Data obtained from the inhibitory test of endophytic bacterial isolates with Cylindrocladium sp. were analyzed using SPSS 25 and further tested by DMRT at the 5% level (0.05), and the characterization results were analyzed qualitatively based on the indicators.

3. RESULT AND DISCUSSION

Test of the inhibitory power of endophytic bacteria against Cylindrocladium sp.

The outcomes of the experiment on endophytic bacterial isolates' ability to inhibit the mycelium of Cylindrocladium sp. indicated that the four isolates (E209, E320, E323, and E327) successfully suppressed the growth of Cylindrocladium sp. mycelium. This inhibitory effect was observed through the formation of an inhibition zone between the endophytic bacterial isolates and Cylindrocladium sp (refer to Figure 1). The assessment of antifungal activity levels revealed that three endophytic bacterial isolates exhibited moderate antifungal activity, while one isolate displayed weak activity (see Table 1). Specifically, only E320 and E323 demonstrated an inhibitory capacity exceeding 30%, prompting further biochemical characterization.

Table 1. Average inhibitory power of endophytic bacteria against Cylindrocladium sp. in vitro(%)

Isolate Code	Inhibitory power (%)		Antifungal Activity Levels*
E309	27.57 ± 11.80	ab	Medium
E320	38.79 ± 6.99	a	Medium
E323	41.25 ± 7.01	a	Medium
E327	15.60 ± 3.92	b	Weak

Note: Numbers followed by the same letter are not significantly different after further testing with DMRT at the 5% level; (R: Rhizosphere, E: Endophyte); *Level of antifungal activity according to Mori et al.1997

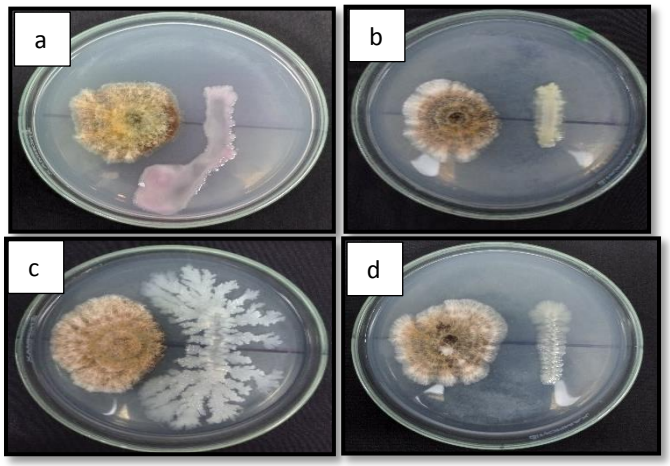

Figure 2. Results of the inhibitory power test for endophytic bacterial isolates; a) E309, b) E320, c) E323 and d) E323

Table 1 illustrates that the four endophytic bacterial isolates exhibited varying degrees of inhibitory efficacy. Notably, Isolate E323 demonstrated the highest inhibitory power at 41.25%, accompanied by a moderate level of

antifungal activity. This is evident from the formation of an inhibition zone, as depicted in Figure 2. Additionally, the growth of the E323 bacterial isolate was observed to be rapid. The formation of inhibition zones by endophytic bacterial isolates is believed to occur through antibiosis and nutritional competition with the fungus Cylindrocladium sp in vitro on petri dishes. Antagonistic bacteria employ various mechanisms, including competition for resources and space, as well as the production of bioactive compounds such as antibiotics, lytic enzymes, hydrogen cyanide, and siderophores (Noumavo et al., 2016). Ernia (2020) reported that Myroides profundi bacteria effectively suppressed the growth of the Fusarium proliferatum fungus through direct antibiosis, facilitated by the production of Hexadecanoic acid, 9-octadecenoic acid, linoleic acid, and piperine. Consequently, this inhibition leads to deformities in the pathogenic hyphae, such as rolling, entanglement, curling, swelling, and even plasmolysis (Widiantini and Fuji, 2020).

The difference in the percentage of inhibitory power obtained indicates that the isolates used were different types of endophytic bacteria, so that the bioactive compounds and antimicrobial concentrations of these bacterial isolates were also different. This is supported by the results of the characterization of two isolates which produced the highest inhibition zones (table 2). Apart from that, it is influenced by the response of the pathogenic fungus to secondary metabolite compounds produced by endophytic bacteria. The results of research by Chen et al., (2009), state that Bacillus endophytic bacteria produce secondary metabolites in the form of surfactin, bacillomycin, fengycin, peptides and iron. the siderophore bacilibactin which can act as an antifungal. Other research from Dunlap et al., (2016) and Guevara – Avendano et al,. (2017) stated that several antagonistic bacteria produce secondary metabolite compounds from the lipopeptide, phenol and flavonoid groups which can suppress pathogenic fungi.

Characterization of selected endophytic bacterial isolates.

Endophytic bacterial isolates that had more than 30% inhibitory power against Cylindrocladium sp were then characterized biochemically. The characteristic results showed that isolates E320 and E323 were gram positive and capable of producing protease activity and only isolate E320 was capable of producing cellulase and siderophores (Table 2). This ability could have potential as a biocontrol agent against Cylindrocldium sp.

Table 2. Results of biochemical characterization of selected endophytic bacterial isolates

Isolate Code	Biochemical Characteristics					
	Gram	Protease (mm)	Chitinase	Cellulase	HCN	Siderofor
E320	Positive	1.63	-	+	-	+
E323	Positive	1.50	-	-	-	-

Description: + sign: capable of producing; sign -: unable to produce

The activity of protease enzymes plays a crucial role in the ability of endophytic bacteria to disrupt the cell walls of pathogenic fungi responsible for plant diseases. Protease enzymes are specifically designed to break down proteins into amino acids. Furthermore, these enzymes have the additional

benefit of promoting plant growth by converting soil proteins into essential nutrients that can be readily absorbed by plant roots. According to Agustin et al. (2022), Klebsiella pneumoniae, Pseudomonas aeruginosa, and Enterobacter asburiae have demonstrated the capacity to impede the growth of Sclerotium rolfsii mycelium through the production of protease, cellulase, and chitinase enzymes.

Isolate E323 exhibited a higher level of inhibition compared to isolate E320, which demonstrated the ability to produce cellulase and siderophore enzymes. This disparity in inhibitory activity may be attributed to the presence of additional metabolite compounds in the E323 endophytic bacteria that are involved in the mechanism of inhibition against the Cylindrocladium pathogen. Vallejo et al. (2020) found that secondary metabolite compounds such as 2-nonanone, 2-undecanone, disulfide dimethyl, and 1-butanol 3-methyl, produced by Bacillus sp., Pseudomonas sp., and Actinobacteria, can act as antifungals. Furthermore, the presence of cellulase enzymatic activity does not consistently correlate with the inhibition of pathogenic fungi. Meliah et al. (2021) reported that endophytic bacteria producing cellulase and protease enzymes exhibit lower inhibitory efficacy compared to those only producing protease enzymes. This is due to the fact that not all fungi have cellulose as a major component of their cell walls, and only a limited number of fungi store this polysaccharide in their cell walls.

4. CONCLUSION

The growth of Cylindrocaldium sp mycelium was effectively suppressed by the four endophytic bacterial isolates. Among them, isolates E320 and E323 exhibited a relatively low inhibitory power of 30%. However, isolate E323 demonstrated a higher potential as a biocontrol agent, with an inhibitory power percentage of 41.25%. Additionally, this isolate also displayed faster growth compared to other bacterial isolates. Both E320 and E323 isolates possess the ability to produce protease activity, which not only contributes to their biocontrol properties but also aids in promoting plant growth..

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