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**Abstract.** This study aimed to screen the endophytic bacteria and to establishe their potential as biological control agents against Antrachnose disease in Cocoa. In-vitro testing through duel culture on PPDA media was carried out to select endophytic bacteria which had inhibitory ability against *C. gloeosporioides* isolates CDKW01. The five best isolates were selected for the activity and antagonistic mechanism against *C. gloeosporioides* isolates CDKS02 and CBKS03. Endophytic bacterial isolates of 2RWB2, 5BPR1, and 3 BPL had very strong inhibitory ability (> 50%) with an antagonistic mechanism in the form of the ability to produce antibiosis compounds, lyse walls and compete with pathogens.

## 1. Introduction

Antrachnose disease caused by *Colletotrichum gloeosporioides* has been identified as a major disease in cocoa planting [1]. The main symptoms of C. gloeosporioides infection in cocoa are the formation of a yellow circle (halo) around the diseased tissues, and the occurrence of curved dead tissues (anthracnose). Plants that are severely affected by this pathogen bear little fruits so that the yield is greatly reduced [2].

Control of this disease has been largely dependent on chemical fungicides inhibiting vegetative pathogen growth. However, the chemical practices to overcome plant disease problem have adverse environmental effects affecting non-target organisms and causing health hazards to humans, besides demanding high costs [3]. Biological agents have been considered as an alternative approach for controlling various plant diseases, one of biological agens is endophyte bacteria.

Studying endophytic bacteria as a biological control agent that suppresses plant diseases has gained much attention in pathological research [4,5]. Endophytic microbes are microorganisms that live in the intercellular spaces of plant for most if not all their life cycles with no pathogenic effects on their hosts [6]. Endophytic bacteria have been isolated from a wide range of hosts, such as palm oil [5] soybean [7], rubber trees [8]. The capability of colonizing host tissue has made endophytes valuable and effective for agriculture as a tool to improve crop performance compared to other biological agents [9]. As an internal colonizer of the root system, endophytes can compete within the vascular system, inhibiting pathogens to obtain both nutrients and space for its proliferation.

Endophytic bacteria, namely *Burkholderia* sp. B212 has the potential to produce secondary metabolites such as pyrrolnitrin [10]. Pyrrolnitrin metabolite of *Pseudomonas* and *Burkholderia* sp. strains were reported to have a strong antifungal activity to control plant diseases against fungal plant pathogens [11]. Thus, this study aimed to screen the endophytic bacteria and to establishe their potential as biological control agents against Antrachnose disease in Cocoa.

## 2. Materials and Methods

### *2.1. Materials*

A total 20 isolates of endophytic bacteria (isolated from cocoa plantation) were used in this studi are collection of Plant Pathology laboratory of Plant Protection, Agriculture Faculty of Halu Oleo University Kendari. *C. gloeosporioides* isolates collected from cocoa plantation in Konawe and Konawe Selatan districts.

### *2.2. Experimental Design*

Experimental design randomized completely design was used in antagonistic assay of 5 superior isolates of endophytic bacteria against isolates CDKS02 and CBKS03 of *C. gloeosporioides* from Konawe Selatan. Each treatmen was repeated 4 times so that there were 40 treatments unit. Data were analyzed using the SAS 9.1.3 version statistical software. The difference of treatment means was separated by Duncan multiple range test at 5% level.

### *2.3. Isolation of C. gloeosporioides*

Pathogenic isolates of *C. gloeosporioides* from Konawe Regency were obtained through isolation from leaves of anthracnose symptomatic cocoa plants, while isolates from South Konawe Regency were isolated from Cocoa leaves and fruit. Symptoms of cacao leaves and fruit were cut in size 1 cm x 1 cm. Symptoms cut into 70% alcohol for 3 minutes then rinsed with sterile distilled water for 3 minutes and dried. The pieces of leaves and fruit were placed separately in a petri dish containing a PDA. Furthermore, cultures in petri dishes were incubated at room temperature. The hyphae of the growing fungus were transferred to a new PDA medium. Growing fungus colonies were identified macrocopically and microscopically then purified and coded.

### *2.4. Endophytic Bacteria Preparationt*

A total of 20 isolates of endophytic bacteria from fruit and healthy cocoa twigs, collections of Phytopathology Laboratory FP-UHO was rejuvenated on solid TSA media in a petri dish using a scratch method and incubated at room temperature for 3 days. Growing bacteria were used for inhibitory testing.

### *2.5 In Vitro Screening of Endophytic Bacteria Against C. gloeosporioides*

In vitro testing of the inhibitory ability of endophytic bacteria against pathogens of *C. gloeosporioides* was carried out through multiple tests. This method was carried out as an initial selection to obtain superior endophytic bacteria isolates as antagonistic agents from 20 isolates of endophytic bacteria from cocoa plants. Purified *C. gloeosporioides* isolates were taken using temporary cork with a diameter of 4 mm, then inoculated over a medium-sized PDA containing petri dish 3 cm from the edge of the cup. A day later vertically inoculated endophytic bacteria with a distance of 3 cm from the edge of the cup in the opposite direction with the location of 1 pathogenic isolate fungi (Figure 1). Petri dishes were incubated at room temperature and observed the inhibitory ability of endophytic bacteria against *C. gloeosporioides* at ages 2, 4 and 6 days after the duel test

The inhibitory ability was calculated by measuring the radius of growth of the pathogenic fungi towards the edge of the petri dish (R1) and the radius of growth of the pathogenic fungus towards the endophytic bacteria (R2). Furthermore, the data obtained was used to calculate the inhibitory ability (DH) of endophytic bacteria isolates against pathogens of *C. gloeosporioides*, that is:

$$\text{Inhibitory ability} = \frac{R1-R2}{R1} \times 100\% \quad (1)$$

*2.6. Antagonistic Assay of Endophitic Bacteria Against C. gloeosporioides*

This test was carried out on the 5 best endophytic bacteria isolates in the screening test. The test used a double culture method with *C. gloeosporioides* isolates CDKS02 and CBKS03 as target pathogens. *C. gloeosporioides* isolates of CDKS02 and CBKS03 were inoculated on a medium PDA petri dish with 3 cm from the edge of the cup separately. One day the endophytic bacteria were inoculated vertically with 3 cm from the edge of the cup in the opposite direction to the location of the pathogenic fungus.

The percentage of inhibitory ability was measured on the 7th day after inoculation using a formula as described previously [8]. The clear zone formed between the two isolates shows the ability of endophytic bacteria to produce antibiosis compounds, while the inhibition without clear zones indicates the antagonistic mechanism occurs through competition. The occurrence of lysis in hyphae of *C. gloeosporioides* through microscopic observation is also an indicator of the antagonistic properties of endophytic bacteria tested.

*2.7. Data Analysis*

Observation data were analyzed using variance. Means of treatment effects were separated using the Duncan's Multiple Distance Test (UJBD) at 95% confidence level.

## 3. Results and Discussion

*3.1. Morphological Identification*

A total of 3 isolates were obtained from cacao leaves and fruit which showed anthracnose symptoms. A total of 2 isolates from cocoa leaves were CDKW01 isolates from Konawe and CDKS02 isolates from South Konawe, and 1 isolate from cacao fruit, namely CBKS03 isolates from South Konawe. The morphology of the three isolates is relatively the same, namely the color of the colony seen above was initially white and changes to grayish white in accordance with the increasing age of the colony. The solid-looking colonies were blackish gray. Conical cylinders with rounded ends, did not form oil bubbles and were not found on the PDA medium (Figure 1). Based on the morphological character, it was identified that the fungus associated with anthracnose symptoms in the cacao leaves and fruit was *Colletotrichum gloeosporioides*.

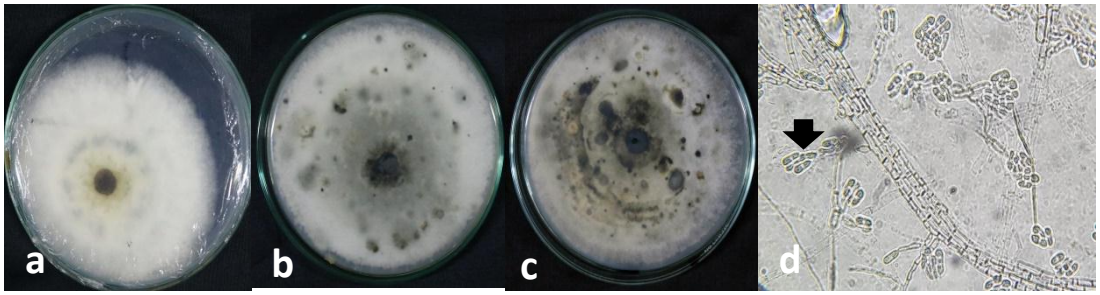


**Figure 1**. *Colletotrichum gloeosporioides*, from above (a dan b), from below (c), and conidium under microscope observation at 400x magnification (d)

*3.2. In Vitro Screening of Endophytic Bacteria against C. gloeosporioides*

The results of multiple culture tests on 20 endophytic bacterial isolates showed that 8 isolates of endophytic bacteria were able to inhibit the growth of C. gloeosporioides CDKS01 isolates more than 50% on the 6th day after inoculation, 5 of 8 endophytic bacterial isolates that had the best inhibitory ability were selected for used in subsequent tests (Table 1).

**Tablel 1**. The results of screening of endophytic bacteria which have very strong inhibitory ability against CDKS01

| No | Isolate code Endophytic bacteria | Inhibitory ability (%) on day ... | | |
|---|---|---|---|---|
| | | 2 AI | 4 AI | 6 AI |
| 1 | 2RWB2*) | 13 | 53 | 69 |
| 2 | 5BPR1*) | 24 | 53 | 62 |
| 3 | 3BPL*) | 19 | 45 | 60 |
| 4 | 3BWB*) | 6 | 33 | 58 |
| 5 | 2RWB*) | 5 | 20 | 56 |
| 6 | 5BRB3 | 11 | 33 | 53 |
| 7 | 2BPR | 10 | 39 | 53 |
| 8 | 3BRB | 16 | 20 | 51 |

Note: *) selected endophytic bacteria; AI = after inoculation

*3.3. Antagonistic Mechanismes of Endophytic Bacteria against C. gloeosporioides*

The test results showed that the five endophytic bacteria tested continued to have inhibitory ability against *C. gloeosporioides* isolates CDKS02 and CBKS03. The isolates 2RWB2, 5BPR1, and 3 BPL had very strong inhibitory ability (> 50%) and were significantly different from isolates 3BWB and 2RWB which had inhibitory ability below 50% (Table 2).

**Table 2**. Inhibitition and antagonistic mechanisms of endophytic bacteria against *C. gloeosporioides*

| No | Code of EB | *C. gloeosporioides* CDKS02 | | | | *C. gloeosporioides* CBKS03 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | % IH | AB | CP | LS | % IH | AB | CP | LS |
| 1 | 2RWB2 | 59.33a | + | + | + | 64.66a | + | + | + |
| 2 | 5BPR1 | 57.33a | + | + | + | 59.33a | + | + | + |
| 3 | 3BPL | 55.00a | + | + | + | 52.66a | + | + | + |
| 4 | 3BWB | 46.00b | - | + | - | 47.33b | - | + | - |
| 5 | 2RWB | 47.33b | - | + | | 50.55b | - | + | - |

Note: IH: inhibitation, EB: Endophytic bacteria, AB: antagonistic, LS: lysis, CP: Competition. Numbers followed by same letters at same column showed no significant different at α 5%.

The results of the observations in Table 2 show that isolates that have a very strong inhibitory effect on *C. gloeosporioides* have antibiosis activity, lysis of *C. gloeosporioides* hyphae, and can compete with pathogens in using space (Figure 2)

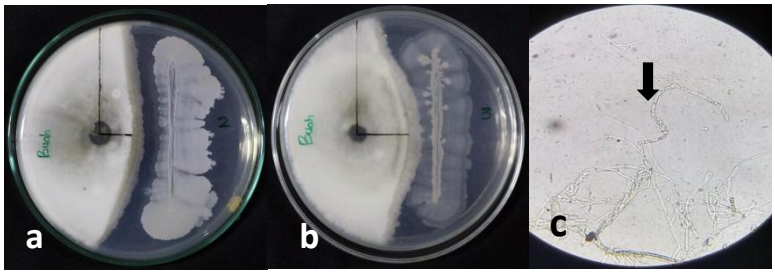


**Figure 2**. Mechanism of endophytic bacterial antagonistic activity against *C. gloeosporioides:* (a)antibiotic activity, (b)competition, (c) hyphae destruction

Antibiotic activity is characterized by the formation of clear zones between endophytic and pathogenic bacteria (Figure 2a), whereas inhibition of the growth of pathogens without clear zones is a sign of the mechanism of competition for space and nutrition (2b). The production of antimicrobial compounds in endophytic bacteria causes lysis characterized by the destruction of hyphae of *C.*

*gloeosporioides* (Figure 2c). These results corroborate the results of previous studies that ME8 and MR3 bacteria which have high inhibitory (> 50%) against *Rigidoporus lignosus*, are able to produce antibiosis compounds and have rapid growth that inhibits the growth of pathogens [8]. Bacillus cereus 11 UJ has inhibitory activity against *P. oryzae* associated with metabolic compounds produced including 19-cyclolanostan-3-ol and acetate [12].

## 4. Conclusion

Of the 20 isolates tested, 8 isolates of endophytic bacteria had very strong antagonistic activity with> 50% inhibitory effect on *C. gloeosporioides* CDKW01 causing anthracnose disease in cocoa. Endophytic bacterial isolates 2RWB2, 5BPR1, and 3 BPL also had > 50% inhibitory effect on *C. gloeosporioides* CDKS02 and CBKS03 isolates with an antagonistic mechanism in the form of the ability to produce antibiosis compounds, lyse walls and compete with pathogens, so that the three endophytic bacteria isolates could potentially be used as anthracnose disease biological agents in cocoa.

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