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Isolation and Evaluation of Endophytic Bacteria From Rubber leaves (*Hevea brasiliensis*) Against Circular Leaf Spot Disease

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Abstract- This study was conducted at the Rubber Research Institute, Dartonfield, Agalawatta, Sri Lanka to identify alternative biological control agents to manage the Circular Leaf Spot Disease in rubber cultivation. The newly reported Circular Leaf Spot Disease caused by the Colletotrichum spp and Pestalotioides group which is distributed in Sri Lanka and also other rubber growing countries in the world. In Sri Lanka, the incidence of the disease is increasingly destructive in rubber plantations. In this study, we isolated endophytic bacteria to assess their antagonistic activity against pathogens causing Circular Leaf Spot Disease (Gunarathne & Fernando, 2017). The endophytic bacteria were isolated from Rubber leaves using 3 clones (RRIC 100,121 and RRISL 203) at bud wood nursery. The isolated endophytic bacteria were analyzed using cultural and microscopic characters. Results revealed that 16 endophytic bacteria were isolated and six strains (Ctr EB1, P44, EB3, Cfr EB4, Cfr EB1, Cfr EB4 and P20 EB5) were identified as the most potent antagonistic endophytic bacteria based on their inhibition percentage. This study highlights the high potential of the isolated highest inhibition percentage endophytic bacteria to combat the circular leaf spot pathogens in Hevea brasiliensis.

Key Word - Endophytic bacteria, Rubber, Circular Leaf Spot Disease, Pathogen

I. INTRODUCTION

Hevea brasiliensis commonly known as rubber tree (Safwan *et al*.,2019), is an economically important estate crop in many Asian countries such as Indonesia, Malaysia, Thailand, India, Sri Lanka, China, and other countries in the world (Jinji *et al*.,2007). Rubber plantation plays a major role in the Sri Lankan economy. Major rubber-growing areas in Sri Lanka such as Kalutara, Rathnapura, Kegalle, and Galle districts are severely affected by most major rubber diseases (Nadeeshani *et al*.,2021). There are many diseases such as leaf disease (Abraham *et al*.,2013), root diseases (Nandris, 1987), panel diseases, and stem diseases noted among rubber cultivation areas (Liyanage,2021). Most of the diseases are caused by fungal pathogens and sometimes the tree is taken up to depth (Safwan *et al*.,2023).

The newly reported Circular Leaf Spot Disease has also severely infected the Sri Lankan rubber sector within the last two years. The leaf disease starts to spread and shows the symptoms of circular, brownish spots of different sizes with the onset of the rains. Later, these spots may expand to form circular brown to silvery spots with a yellow halo (Alchemi & Jamin, 2022).

Brown circular lesions (typical symptom), yellow large irregular lesions, slightly green circular patches, mall irregular brown patches, brown irregular lesions, brown pinpoint lesions and brown pin head lesions also show as other symptoms (Narayanasamy,2013). Circular Leaf Spot Disease, which is a new threat to rubber cultivation around the world, has become a serious challenge to the Sri Lankan rubber industry (Aliya, 2022). The first pandemic condition of this disease had been reported in Malaysia in 2017 – 2018 and later, then after the disease spread to Indonesia as well destroying many rubber clones (Hassan *et al.*, 2013).

Therefore, the current study is focused on the isolation and selection of the most effective antagonistic endophytes to use as a biological controlling agent to control the Circular Leaf Spot Disease.

Isolation of endophytic bacteria is mainly targeted from the major rubber clones growing in Sri Lanka; RRIC 100, RRIC 121, and RRISL 203.

II. MATERIALS AND METHODOLOGY

A. Research area

This study was conducted at the plant pathology and microbiology laboratory of Rubber Research Institute, Dartonfield, Agalawatta, Sri Lanka.

B. Collection of Leaf samples

Healthy rubber leaves in mature stage (hardened straighten dark green leaves from lateral branches) were collected from 3 clones (RRIC 100, RRISL 203, RRIC 121). Five plants were selected from each clone and six leaflets from each plant were selected. The Randomize Complete Block Design (RCBD) was applied for the isolation of "Endophytic Bacteria".

C. Isolation of endophytic bacteria

The outer surfaces of leaflets were cleaned by running tap water. Subsequently, they were cut into small pieces and sterilized the surface with 70% ethanol for 3 minutes. The sterilized leaf pieces were washed in sterilized distilled water series. They were blotted using sterilized filter papers. After that, the leaf pieces were placed on PDA culture media and they were incubated at 28°C for 3 days. Three Petri dishes were used for each leaflet. Morphologically different bacterial colonies arising from the inoculated leaf pieces were separated after 3 days. A total number of eight endophytic bacteria from RRIC 121 and five from RRISL 203 and three from RRIC 100 were isolated.

D. Preparation of pure cultures of endophytic bacteria

The inoculating loop (heating to red hot to destroy contaminating bacterial spores) was
sterilized by using a burner flaming and allowed to cool. After that, small portions were picked from isolated colonies. Streaking was done by taking a single colony of bacteria to obtain pure cultures. After that, they were spread in a zig-zag manner on PDA containing Petri dishes using the Zig-Zag streaking method.

E. Preparation of previously isolated pure cultures of main pathogens in circular leaf spot disease.

Colletotrichum spp. (Six species)-
C. acutatum, C.siamense, C. fructicola,
C. tropicale, C. gigasporum, C. gloesporiodes

Pestalotioides group-
P7(Pestalotiopsis sp.),
P44 (Neopestalotiopsis sp.),
P20 (Pseudopestalotiopsis sp.)

F. Screening isolated organisms against the pathogens to reveal antagonistic ability; Pestalotioides group and Colletotrichum spp.

The percentage inhibition of each pathogen by the endophytic bacteria was calculated using the following equation.

$$\text{Inhibition (\%)} = (R1 - R2)/R1 \times 100$$

R1 = the radius of control plate
R2 = the radius of dual culture plate

The highest inhibition percentage against 9 fungal pathogens were evaluated and the best isolates with high percentage of inhibition was selected using the above calculated data and graphs.

G. Morphological characterization of selected endophytic bacteria

The morphology observation of endophytic bacteria was including Cultural and Microscopic Characterization.

H. Preservation of bacterial cultures

Glycerol Stock (Long term preservation)

A broth culture was prepared and isolated endophytic bacteria were inoculated into the broth culture. After that, it was kept for several days for bacteria growth. Next, 20% Glycerol stock (20% Glycerol+ 80% Distilled water) was prepared and 500 µl solutions were taken and put into Eppendorf tubes. All the Eppendorf tube was autoclaved. After that, 500 µl of broth was put into glycerol contain Eppendorf tubes. Finally, the tubes were sealed using parafilm and stored in the freezer.

I. Experimental design

In this experiment, Healthy rubber leaves in the mature stage were used. The experiment adopted a Complete Randomized Design (CRD) with 3 replicates and 3 treatments from RRIC 100, 5 treatments from RRISL 203, and 8 treatments from RRIC 121.

J. Statistical analysis

Data were subjected to statistical analysis of variance by using SAS statistical package.

III. RESULTS AND DISCUSSION

The current study was carried out to identify the potential bio-controlling agents to control Circular Leaf Spots Disease which had been identified in the recent past as a severe disease in *H. brasiliensis* in Sri Lanka. The investigation of the top antagonistic endophytic bacteria isolated from the three important rubber clones, including RRIC 100, RRISL 203, and RRIC 121, was the main objective of the study. Below, the results were discussed to the study's goal.

A. *Isolation of endophytic bacteria*

A total of 16 endophytic bacteria with different morphological characteristics were found, including 3 strains from RRIC 100, 5 strains from RRISL 203, and 8 strains from RRIC 121.

B. *Pure cultures of main pathogens in causing Circular Leaf Spot Disease*

The main causative agent has been identified as the *Colletotrichum* spp. while the three *Pestalotioides* groups viz. *Pestalotiopsis*, *Neopestalotiopsis* and *Pseudopestalotiopsis* have been identified as the secondary invading group.

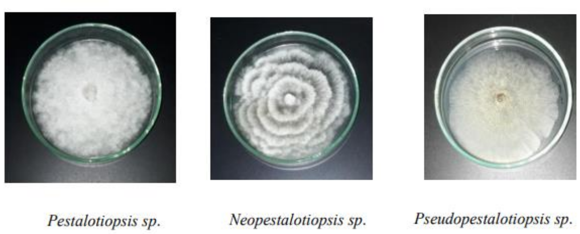

Figure 1: *Pestalotioides* groups

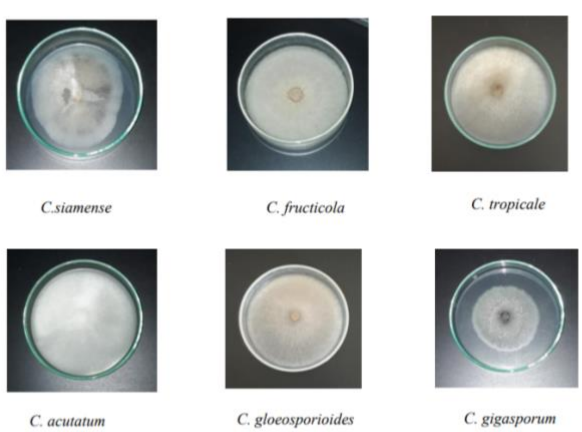

Figure 2: *Colletotrichum* spp.

After 7 days of incubation at room temperature, pure cultures of each isolate displayed the following cultural characteristics in Potato Dextrose Agar medium (Figure: 1 and Figure: 2).

All the isolates were evaluated for their antagonistic activity in vitro against the Circular Leaf Spot Disease by using the dual culture technique (Fernando, 2010). As a result, the percentage inhibition was used to choose the potential isolates. In a completely randomized design, each test was carried out three times. The best six endophytic bacterial strains (from RRIC 100, RRISL 203, and RRIC 121) were chosen for the dual culture experiment based on the findings of the analysis of variance. The cluster diagram contained all of the final results of the analysis of variance for the various clones.

1) *RRIC 100*

From inhibition percentages in RRIC 100 clone, showed the best inhibition percentages showing group and that has the highest inhibition percentage of endophytic bacteria against the pathogens of Circular Leaf Spot Disease compared with the other of the RRIC 100 clone.

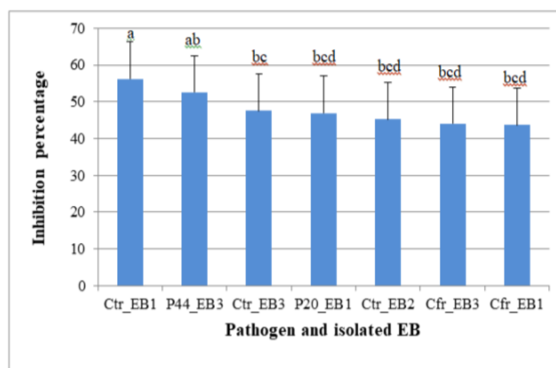

Figure 3: Inhibitory powers of endophytic bacterial isolates on RRIC 100

The Ctr EB1 (56.250%) and P44 EB3 (52.574%) - (EB1, EB3) were chosen as the best antagonistic endophytic bacteria of RRIC100 after taking into account the entire group. The antagonistic effect of the top two endophytic bacteria in RRIC 100 was demonstrated in comparison to control culture and dual culture plate. Several endophytic bacterial isolates that positively affect for suppression of the causative pathogens were able to identify based on the percentage inhibition of the pathogen.

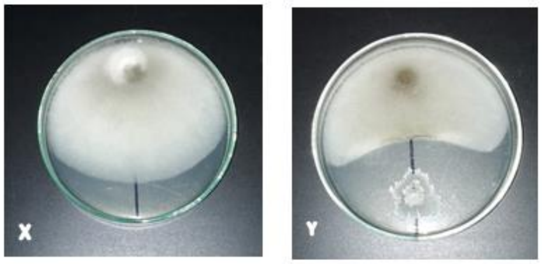

Figure 4: X- Control, Y- Antagonistic effect of EB1 against *Colletrichum tropicale*

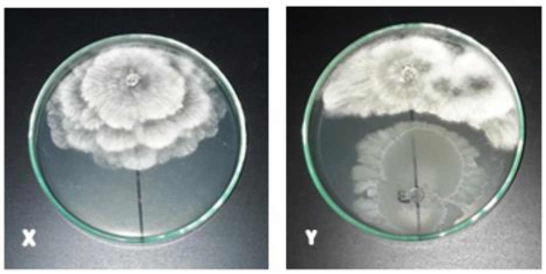

Figure 5: X- Control, Y- Antagonistic effect of EB3 against *Neopestalotiopsis* sp.

2) RRISL 203

Out of the five endophytic bacteria that were isolated from the RRISL 203 clone, Figure 6 revealed the best inhibition percentages, with the best among all other isolates.

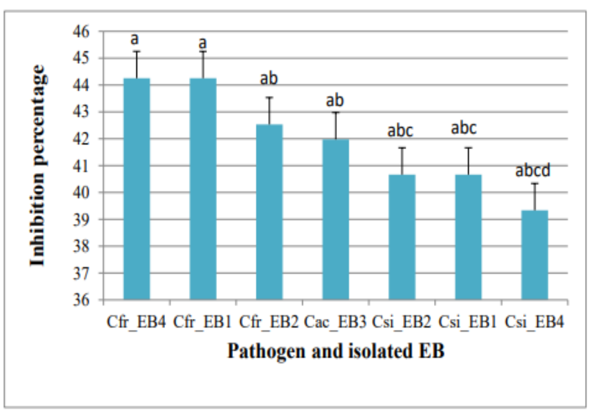

Figure 6: Inhibitory powers of endophytic bacterial isolates on RRISL 203

The Cfr EB4 (44.253%) and Cfr EB1 (44.253%) - (EB4, EB1) were chosen as the best antagonistic endophytic bacteria from the out of all the data of the RRISL 203 clone. Figure: 7 exhibited that two of the best endophytic bacteria had an antagonistic effect in RRISL 203 when compared to the control culture.

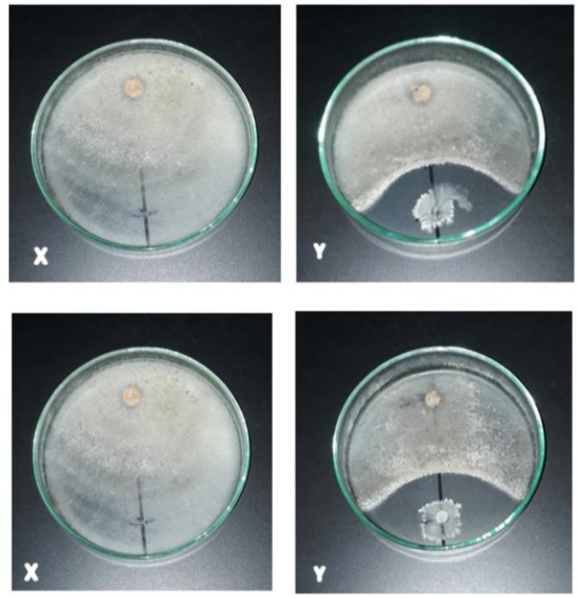

Figure 7: X- Control; Y-Antagonistic effects of EB1 and EB4 against *Colletotrichum fructicola*

3) RRIC 121

The most endophytic bacteria (8 bacterial isolates) were isolated from this clone. The isolates with the highest inhibitory percentages were designated as the Cfr EB4 (42.949%) and P20 EB5 (41.958%). Comparing the all-other isolates from the RRIC 121 clone, EB4 and EB5 contains the largest inhibition percentage of endophytic bacteria that are inhibited pathogens against the disease from this clone. Figure: 8 exhibited the antagonistic effect of the two best endophytic bacteria in RRIC 121 when compared to control culture.

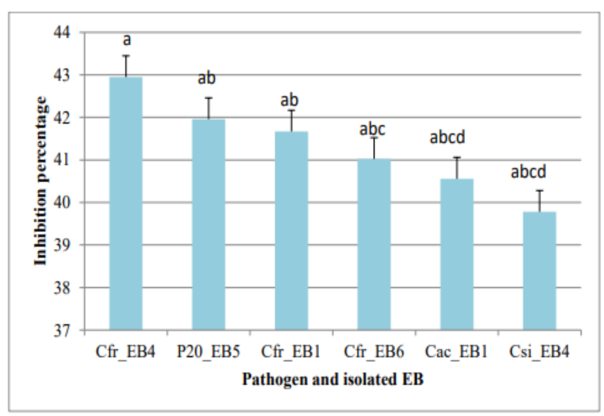

Figure 8: Inhibitory powers of endophytic bacterial isolates RRIC 121

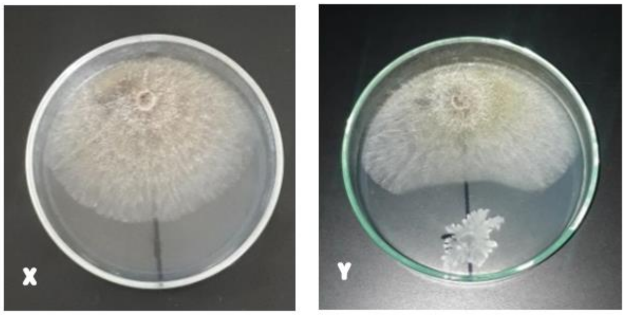

Figure 9: X- Control; Y-Antagonistic effects of EB5 against *Pseudopestalotiopsis* sp.

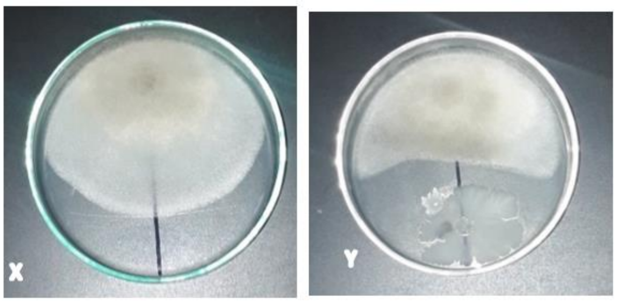

Figure 10: X- Control; Y-Antagonistic effects of EB4 against *Colletotrichum fructicola*

The findings from the analysis of variance revealed that the use of endophytic bacteria significantly inhibited the pathogen that causes Circular Leaf Spot Disease under in vitro conditions. Regardless of the clone, the best antagonistic bacteria from all the isolates were chosen as 56.250% (Ctr EB1).

Table 1: Morphological characteristics of six best antagonistic endophytic bacteria

Characteristics	**RRIC 100-EB1**	**RRIC 100 EB 3**	**RRISL203 EB 1**	**RRISL203 EB 4**	**RRIC 121 EB 4**	**RRIC 121-EB5**
Shape	Irregular	Irregular	Irregular	Filamentous	Irregular	Filamentous
Elevation	Flat	Flat	Raised	Flat	Flat	Flat
Margin	Lobate	Undulate	Undulate	Filamentous	Lobate	Filamentous
Texture	Shiny, Viscous	Matte, Brittle	Matte, Brittle	Shiny, Viscous	Shiny, Viscous	Slimy, Moist
Color	White	White	White	White	White	White
Gram (+/-)	Gram (+)	Gram (-)	Gram (-)	Gram (-)	Gram (-)	Gram (-)

The resulting endophytic bacterial colonies include a variety of morphological characteristics, including filamentous and irregular forms. The majority of the recovered bacteria had elevated elevation, while the remaining isolates have flat elevation. The findings of gram staining can be determined from bacteria isolated that fall into the gram-positive and gram-negative categories. Regarding their microscopic properties, the outcome of the characterization of isolated endophytic bacteria varied substantially from isolate to isolate. It can be inferred from these variations that there are multiple types. Pathogenic fungi are typically inhibited by antagonistic bacteria via a variety of ways (Simbolon *et al*., 2022). The pathogenic fungi's structural constituents can be harmed by bioactive chemicals produced by antagonistic bacteria. Hydrolytic enzymes, such as chitinase produced by chitinolytic bacteria, can harm the structural elements of fungi. These bioactive molecules may also alter the permeability of the membranes in fungal cells, which would interfere with the movement of elements required for metabolism. Inhibitors of an enzyme in the fungus can be created by bacteria-produced substances. The enzymatic activity of the cell will be interrupted if the fungus enzyme plays a significant role in metabolism, which would lower fungal growth.

Six isolated endophytic bacteria in this study demonstrated high levels of antagonism (41–56%) against the pathogens of Circular Leaf Spot Disease in dual culture technique, compared to the other endophytic bacteria isolates, out of 16 isolated bacteria within 3 clones (RRIC 100, RRISL 203, and RRIC 121). The capacity of these six of the 16 endophytic bacteria isolates to inhibit the Circular Leaf Spot Disease pathogen was determined by the dual culture plates' rate of inhibition relative to the control culture. Endophytic bacteria function as biological agents (Daulagala, 2021) that create antimicrobial substances to combat pathogens, and growth regulators, fix nitrogen, and mobilize phosphate, which helps to promote and reinforce the development of plant resistance (Medison *et al*., 2022). Researchers found that bacteria caused mycelium to morphologically change significantly, destroy the fungal cell wall, prevent conidia from developing normally, and reduce pathogenicity (Idiyatov *et al*., 2021). Endophytic bacteria can contend with phytopathogenic fungi that have developed systemic resistance (Muthukumar *et al*., 2019).

However, further research must be done on the other inhibitory substances produced by these six best endophytic bacteria. At present, the disadvantages of chemical pesticides are prominent and the development of environmentally friendly biocontrol agent is needed. The Circular Leaf Spot Disease in rubber caused by *Colletotrichum* spp. and *Pestalotioides* group can be controlled by using endophytic bacteria species as bio-controlling agents, according to this study. The competing bacteria can produce bioactive substances that weaken the pathogenic fungi's structural elements. The agriculture sector is currently advancing towards environmentally sustainable development holding the increase in productivity along with the protection of the natural resource base for future generations. The findings of the studies allow us to draw the conclusion that, when compared to all isolated endophytic bacteria, the selected isolates of EB1 (56.250%) and EB3 (52.574%) from RRIC 100, EB4 (44.253%) and EB1 (44.253%) from RRISL 203 and EB4 (42.949%) and EB5 (41.958%) from RRIC 121 have high antagonistic activity against pathogen of Circular Leaf Spot Disease.

IV. CONCLUSIONS

Sixteen different endophytic bacteria isolates were extracted from different clones in a bud wood nursery in Dartonfield, Agalawtta, and all the laboratory work was carried out at the Plant Pathology and Microbiology Department of the Rubber Research Institute of Sri Lanka. Among all isolates, five isolates were shown significantly higher growth inhibition percentage compared to others under in vitro conditions. The findings of this study showed that different endophytic bacteria isolates extracted from different 3 clones had the ability to control Circular Leaf spot Disease. However, studies are necessary to get conclusive results of the bio efficacy of endophytic bacteria as biocontrol agent against Circular Leaf Spot Disease under greenhouse and field conditions.

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