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Potential biocontrol of endophytic fungi against *Lasiodiplodia pseudotheobromae* causal agent of cocoa dieback on cocoa seedling

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Abstract. Endophytic fungi associated with cocoa are one of the effective agents to control plant pathogens on cocoa, including the fungus *Lasiodiplodia pseudotheobromae*. *L. pseudotheobromae* cause a significant disease on cocoa, cocoa dieback. The disease is considered an emerged disease on cocoa in Sulawesi, Indonesia. The research aimed to determine the effect of the endophytic fungi on *L. pseudotheobromae* on cocoa seedlings. This study used a randomized block design consisting of 6 treatments and 3 replications. Five different fungi isolates were used that belong to *Aspergillus* genera. The treatment is three isolates of endophytes fungi (isolate T2S2LT, T245LT, T645LT), *Aspergillus* isolates combination, and control (with and without pathogen). The results showed that the combination of isolates and T645LT isolate was the best treatment for suppressing necrotic symptoms (leaf spot) incidence and severity. Also, T645LT isolate performed well to inhibit leaf blight symptoms incidence severity. Meanwhile, the chlorotic symptom was reduced by isolates combination, followed by T2S2LT, T645LT, and T245LT. Endophytic fungi can potentially be an important option to control cocoa dieback disease.

1. Introduction

Endophytic fungi are promising agents of biological control to suppress the disease and the pathogen. Studies have identified fungal endophytes can effectively reduce various destructive diseases on cacao like black pod rot, frosty pod rot, witches' broom, and vascular streak dieback [1,2,3,4,5,6,7,8,9,10].

Cocoa dieback caused by the ascomycete fungus *Lasiodiplodia* species [11,12,13,14,15,16], one of the pathogen species is *Lasiodiplodia pseudotheobromae* [15,16]. The disease is a new emerging disease in cocoa in Sulawesi, Indonesia [15]. *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous, a member of botryosphaeriaceae, one of the species of the genus *Lasiodiplodia* which is a cosmopolitan fungus that can cause diseases on the plant such as leaf spot, dieback, and canker which can lead to plant death [17,18,19].

The present research using endophytic fungi from stems and leaves of different cocoa genotypes isolated from South Sulawesi, Indonesia [20]. The research aimed to determine the effect of the endophytic fungi on *L. pseudotheobromae* on cocoa seedlings.



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2. Methods

2.1. Preparation of seedling and endophytes application

The Seedling was developed from a popular clone in Sulawesi, Masamba cocoa clone 02 (MCC-02). Cocoa seedlings were prepared inside a mini-house with poly-ethylene (PE) plastic as a roof and wall and placed inside a screen house with half its wall by wire mesh located in Makassar, South Sulawesi Province, Indonesia. Endophytic fungi and the fungus *pseudotheobromae* inoculation was carried out in the afternoon onto 1.5-month-old seedlings. Three isolates of endophytes fungi were isolated from cocoa stems tissue, the fungi belong to *Aspergillus* genera. There are six treatments, three isolates were applied separately, combination of *Aspergillus*, and control (with and without pathogen). The concentration of 39.6×10^6 , 65.05×10^6 , 58.5×10^6 spores/mL of endophytes fungi were sprayed and followed by 0.6×10^6 spores / mL of *L. pseudotheobromae*. Inoculation of *L. pseudotheobromae* was carried out 1 week after inoculation of endophytic fungi. Negative control was only inoculated with sterile aquadest without *L. pseudotheobromae* spores.

2.2. Evaluation

The symptom of the disease was calculated by its incidence and severity at 5 weeks after inoculation. The incidence was measured by calculating the proportion of plants with any infection symptom (the number of infected trees divided by all sampled trees). The symptoms of infected leaves that are attacked by pathogens can be seen by necrotic (leaf spot) and leaf blight) and chlorotic symptoms which appear on the leaves. The incidence of the symptoms was calculated using the following equation:

$$I = \frac{n}{N} \times 100\% \quad (1)$$

where,

I : Incidence of disease
n : Number of symptomatic leaves
N : Number of leaves observed

The disease severity was calculated using the equation as follows [21]:

$$I = \frac{\sum (n \times v)}{N \times Z} \times 100\% \quad (2)$$

Where n represents the number of infected leaves on each score, v is a score on each infestation category, Z is the highest score and N represents the total number of leaves observed. The disease severity was assessed by scoring disease severity in individual leaves as follows: 0 = No visible symptoms on leaves; 1 = < 10% of leaves infected area (leaf shows necrotic lesions and chlorosis); 2 = > 10% - <25% of leaves infected area; 3 = > 25% - <50% leaves infected area; 4 = > 50% - 100% leaves infected area.

2.3. Statistical analysis

an analysis regarding symptoms incidence and severity at five weeks after inoculation were determined using analysis of variance (ANOVA) and standard error. When significant differences are detected, the data is further tested using Tukey's test at the 5% probability level.

3. Results and discussion

The result of the research showed that there were three different symptoms were appeared including necrotic (leaf spot and leaf blight) and chlorotic during the study both on the treatments and on the control after inoculation of the pathogen *L. pseudotheobromae*.

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3.1. Occurrence of necrotic symptoms (leaf spot and leaf blight)

Leaf spot and leaf blight symptoms showed variable incidence and severity among the endophyte isolates. There is an influence of the endophyte fungi isolates on the leaf spot and leaf blight incidence and severity where the influence was highly significant (table 1, 2).

Table 1. Disease incidence and severity inoculated by *L. pseudotheobromae* and endophytic fungi according to necrotic symptoms on leaves (Leaf spot).

Disease evaluation	Necrotic symptoms (leaf spot)					
	Control		Treatment			
	with pathogen	without pathogen	Isolate T2S2LT	Isolate T245LT	Isolate T645LT	Isolates combination
Incidence	31.98 ^c	0.00 ^a	13.07 ^b	14.18 ^b	5.08 ^{ab}	4.94 ^{ab}
Severity	8.12 ^b	0.00 ^a	2.71 ^a	4.18 ^{ab}	1.08 ^a	1.21 ^a

Note: The numbers followed by the same letters in the same row are not significantly different in the Tukey HSD level test of 0.05.

A combination of isolates was the most effective treatment to reduce leaf spot incidence (4.94%), and followed by T645LT (5.08%), T2S2LT (13.07%), and T245LT (14.18%). However, all isolate treatments were not significantly different. Meanwhile, all isolate treatments had significantly different to control with pathogen while control without pathogen showed no symptoms (table 1).

Isolate T645LT showed the best isolates to inhibit the severity of the symptom of the leaf spot with only 1.08% of severity, followed by the combination of isolates (1.21%), T2S2LT (2.71%), and T245LT (4.18%). All endophyte fungi isolate treatments were not significantly different. However, all isolate treatments had significantly different to control with pathogen while control without pathogen showed no symptoms (table 1).

Table 2. Disease incidence and severity inoculated by *L. pseudotheobromae* and endophytic fungi according to necrotic symptoms on leaves (Leaf blight).

Disease evaluation	Necrotic symptoms (leaf blight)					
	Control		Treatment			
	with pathogen	without pathogen	T2s2lt	T245lt	T645lt	Isolates combination
Incidence	53.49 ^b	11.39 ^a	17.34 ^a	18.82 ^a	12.33 ^a	24.51 ^a
Severity	16.05 ^b	2.19 ^a	3.41 ^a	5.37 ^a	1.97 ^a	4.69 ^a

Note: The numbers followed by the same letters in the same row are not significantly different in the Tukey HSD level test of 0.05.

T645LT Isolate was the most effective isolates decrease leaf blight incidence and severity, 12.33% and 1.97%, respectively while T2S2LT was the second most effective isolates followed by T245LT isolate and isolates combination. However, all isolate treatments were significantly different to control with pathogen while control without pathogen showed blight symptoms as well in low percentages (table 2).

3.2. Occurrence of chlorotic symptoms

Chlorotic symptoms indicated different incidence and severity among the endophyte isolates. However, there is no influence of the endophyte fungi isolates on the incidence and severity of the chlorotic symptoms (table 3).

Table 3. Disease incidence and severity inoculated by *L. pseudotheobromae* and endophytic fungi according to chlorotic symptoms on leaves.

Disease evaluation	Chlorotic symptoms					
	Control		Treatment			Isolates combination
	with Pathogen	without Pathogen	T2S2LT	T245LT	T645LT	
Incidence	4.17	1.11	0.00	5.56	1.85	0.00
Severity	2.08	0.56	0.00	2.08	1.39	0.00

A combination of isolates and T2S2LT isolate were the most effective treatments to inhibit the appearance of chlorotic incidence and severity, no symptoms were recorded. The second most effective was T645LT isolate, followed by T2S2LT (5.56%). However, all treatments had no significant difference to control with pathogen and control without pathogen (table 3).

3.3. Discussion

The current study showed endophytic fungi from cocoa stems tissue [20] can inhibit one of the causal agents of cocoa dieback, *L. pseudotheobromae*, on the cocoa seedling. The results indicated that all the isolates of the endophytic fungi completely inhibited three different symptoms produced by *L. pseudotheobromae*, i.e. leaf spot, leaf blight and chlorosis. Although, chlorosis incidence and severity was not significantly different to control, but the endophytic fungi isolates remain lower than the control seedling.

Endophytic fungi was used belong to *Aspergillus* species. *Aspergillus* species have been reported as endophytes with antifungal activity [22, 23] and able to produce many secondary metabolites such as alkaloids, terpenoids [24]. Endophytic fungi associated with cocoa able to protect cocoa plants against phytopathogens through a number of inhibition activities, including competition, antibiosis, and mycoparasitism [4]. Moreover, the endophytic fungi from cocoa tree tissue able to colonized the cocoa plant tissue [4, 10, 25] and able to respond to abiotic and biotic stresses in the plant through induction of some genes [26].

4. Conclusion

Disease incidence and severity caused by the fungus *L. pseudotheobromae* was effectively reduced by endophytic fungi from cocoa stems and leaves tissue applied on seedling. However, a larger study area to provide more data, particularly in the field.

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