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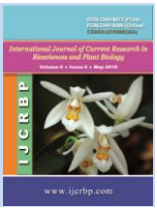

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Endophytic Fungi with Potential as Biological Control Agents for Black Pod Rot Disease [*Phytophthora palmivora* (Butler) Butler]

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ABSTRACT

Production and cocoa plantation area in Indonesia decline every year, major causes is the outbreak of the cocoa black pod disease. Results showed that the antagonistic fungi found based on the DNA sequences are *Lasiodiplodia euphorbicola*, *Diaporthe tectonae*, *Colletotrichum siamense*, *Aspergillus flavus*, *Candida parapsilogis*, and *Aspergillus sydowii*. The results of the best inhibition shown by the endophytic fungus *L. euphorbicola*, thus passed field trials which showed that the higher the concentration of spore suspension the better the power suppress fungal antagonists against *P. palmivora.* The best concentration that colonies of the Petri dish was dissolved in 150 ml of sugar solution (10%).

Introduction

Cocoa plantation area in Indonesia in 2012 - 2015, continue to decline from 1,774,46 in 2012 to 1,704,982 ha in 2015. Declining in area coverage resulted in decreasing number of producing per year, from 740,513 to 701,229 tones (Directorate General of Plantation, 2014). The decline was caused by the addition of land conversion is also one of the reasons is due to interference cocoa pod disease caused by *P. palmivora*.

Endophytic fungi isolated from healthy cocoa plant tissue provides new hope in biological control of cacao pod disease. The research by Mejia et al. (2008) 40% (21/52) of isolated endophytic fungi capable of controlling *P. palmivora*. One fungus findings antagonistic with simple competition mechanism was *Trichoderma* sp. This fungus can competitively inhibit pathogens and antibiosis.

Fungi are known to colonize certain plants with

varying degrees and in varying patterns. While some endophytic fungi in part of this material (Amadi, 2005).

Endophytic fungi especially asexual, endophytic grasses generally seen systemic in collaboration with the plant, mainly through the work of mycotoxins such as alkaloids in the infected grass, which protect host plants from herbivores. Very much evidence for defensive mutualism concept originated from cultivars of grass agronomic studies, which may form endophytic-host interaction (Faeth, 2002).

Endophytic fungus to grow which indicates that the endophyte was found in all plants, in extreme plentiful and often very diverse (Arnold et al., 2002). Very much endophyte derived from internal local infections on leaves, roots, stems and bark, as well as horizontally transferred through spores (Faeth, 2002).

Endophytic fungi can secrete mycotoxins or modify the host's physiology and morphology. Endophytic mycotoxins beneficial for host plant timber as a "defense that is able to trigger" face a host insect herbivores and grass as a "desired plant defense" facing both vertebrate and invertebrate herbivores. Endophyte can also change the other physiology, developmental or morphological characteristics of host plants such as changes in the ability of competition, especially in encountering stress on the environment (Malinowski et al., 1999).

Materials and methods

Place and time of research

The research was conducted in two places, namely 1) on the ground in the form of looking for healthy plant specimens in the form of leaves, stems and fruit, which is located in Perean village, Tabanan, and 2) Laboratory of Biotechnology, Faculty of Agriculture, University of Udayana, from March to August 2017.

Test of inhibition ability of endophytic fungus *in vitro*

Endophytic fungi are found each tested for inhibitory to the growth of pathogenic fungi (*P. palmivora*) with a dual culture technique (in a Petri dish grown each one fungal pathogen flanked by two endophytic fungi). Inhibition ability can be calculated as fellows (Dollar, 2001; Mojica-Marin et al., 2008):

$$\text{Inhibition ability (\%)} = \frac{A - B}{A} \times 100$$

Where: A = *P. palmivora* colony diameter in a single culture (mm);
B = *P. palmivora* colony diameter in a dual culture (mm).

Antagonistic test *in vivo*

In vivo test of antagonistic endophytic fungi were found by pricking with a needle spelden fresh fruit as much as 20 times, then smeared with antagonistic fungus spores (one Petri dish in 250 ml of sterile distilled water). Finally fruits were dipped in a spore suspension of pathogenic fungi (*P. palmivora*). The treatment of fungal antagonists, include:

A = control (without smeared with antagonists without pathogens)
B = 1 antagonist treatment (5×10^7 spore suspension)
C = 2 antagonist treatment (5×10^7 spore suspension)
D = 3 antagonist treatment (5×10^7 spore suspension)
E = 4 antagonist treatment (5×10^7 spore suspension)
F = 5 antagonist treatment (5×10^7 spore suspension)
G = 6 antagonist treatment (5×10^7 spore suspension)
H=Control (without smeared antagonist with pathogens)

All treatments were repeated for 5 times. The experiment was designed with a randomized block design (RBD), and after analysis of variance (ANOVA) followed by the least significant difference test (LSD) at 5% level. Attack parameters measured by the formulation: how punctures are attacked by fungi shared with the entire puncture (20 ×) times 100%.

Antagonistic test in field

Best antagonist been tested in field by spraying into fruit still attached to the tree. The suspension was made by mixing the best of endophytic fungal colonies into a 10% sugar solution. The treatments were to plot (cacao tree) is as follows:

A = Control (without treatment).
B = Treatment with a suspension of spores of the Petri dish/150 ml of sugar solution (10%).
C = Treatment with a spore suspension of a Petri dish/200 ml of sugar solution (10%).
D = Treatment with a spore suspension of a Petri dish/250 ml of sugar solution (10%).
E = Treatment with a spore suspension of a Petri dish/500 ml of sugar solution (10%).
F = Treatment premises spore suspension of the Petri dishes/1000 ml of sugar solution (10%).

All treatments were repeated for 6 times. The experiment was designed with a randomized block design (RBD), and after analysis of variance (ANOVA) followed by the least significant difference test (LSD) at 5% level.

Results

Best endophytic fungi identification by PCR and sequencing

Results amplification 1.2% agarose gel electrophoresis in 1x TAE buffer at a voltage of 50 volts for 30 minutes. DNA bands seen above UV Transilluminator. That generate a DNA fragment size of ± 600 bp (Fig. 1). Furthermore, the DNA fragment was sent to PT Macrogen Inc. Korea to do the nucleotide bases tracing the identity of the fungus.

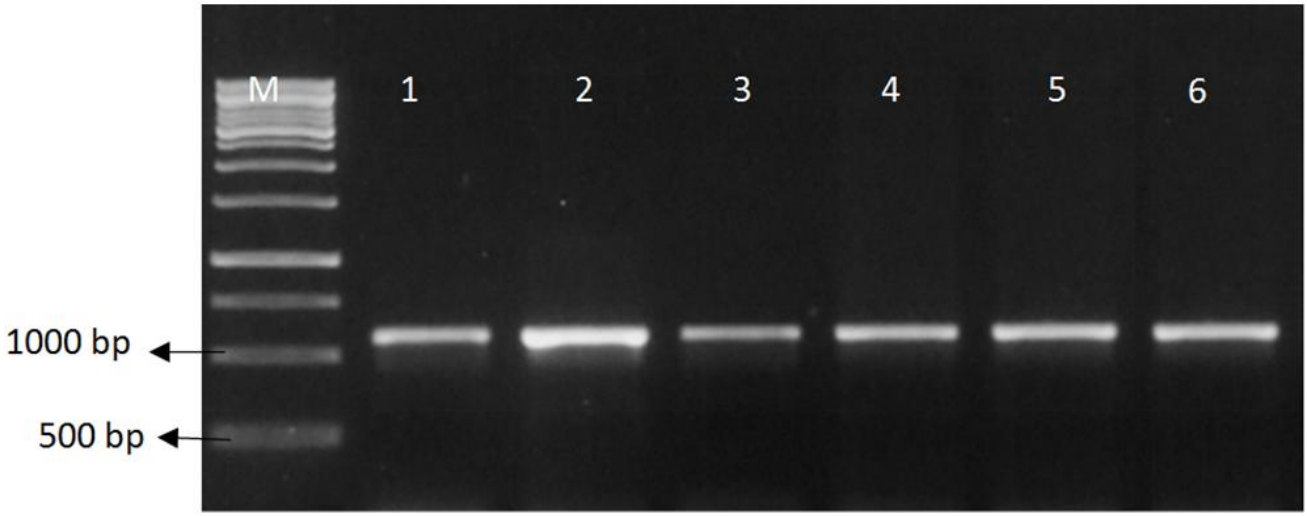

Fig. 1: The DNA fragment of each isolate (M = 1 kb DNA leader, 1, 2, 3, 4, 5 and 6 isolates respectively).

The result of the sequence of nucleotide bases supported by bioinformatics services derived isolates 1 (of endophyte best rod 1) was *Lasiodiplodia euphorbicola*, isolates 2 (origin endophyte best rind 2) was *Diaporthe tectonae*, isolate 3 (origin of the best leaf endophytic 3), namely *Colletotrichum siamense*, isolate 4 (origin endophyte best rod 4) was *Candida parapsilogis*, and isolates 5 (origin of best leaf endophytic) was *Aspergillus sydowii* and isolates 6 (origin endophyte best rind 6) was *Aspergillus flavus*.

Inhibition ability test

Inhibition ability test for six endophytic fungi on the growth of *P. palmivora in vitro* studies suggest that endophytic stem 3 (*L. euphorbicola*) with 91.11% inhibition, followed by endophytic rind 4 (*D. tectonae*) and endophytic leaf 2 (*C. siamense*) respectively by 66.66%, then endophytic rind 3 (*A. flavus*) amounted to 65.92%, endophytic stem 2 (*C. parapsilogis*) amounted to 62.96% and 55.55% for leaf endophytic 5 (*A. sydowii*) (Figs. 2 and 3).

Antagonistic test *in vivo*

Test antagonist *in vivo* indicated that the treatment with the antagonist fungus, *L. euphorbicola* gave the best results among the other treatments with *D. tectonae*, *C. siamense*, *A. flavus*, *C. parapsilogis*, *A. sydowii*), with a disease incidence of 0%, followed by *D. tectonae* by 25%, *C. siamense* by 35%, then *A. flavus*, *A. sydowii*, and *C. parapsilogis* 100% respectively (Table 1, Fig. 4).

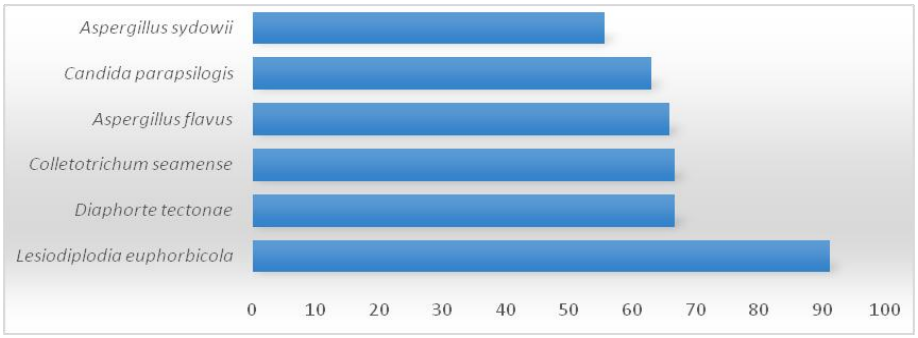

Fig. 2: Inhibition ability of endophytic fungus on *P. palmivora in vitro.*

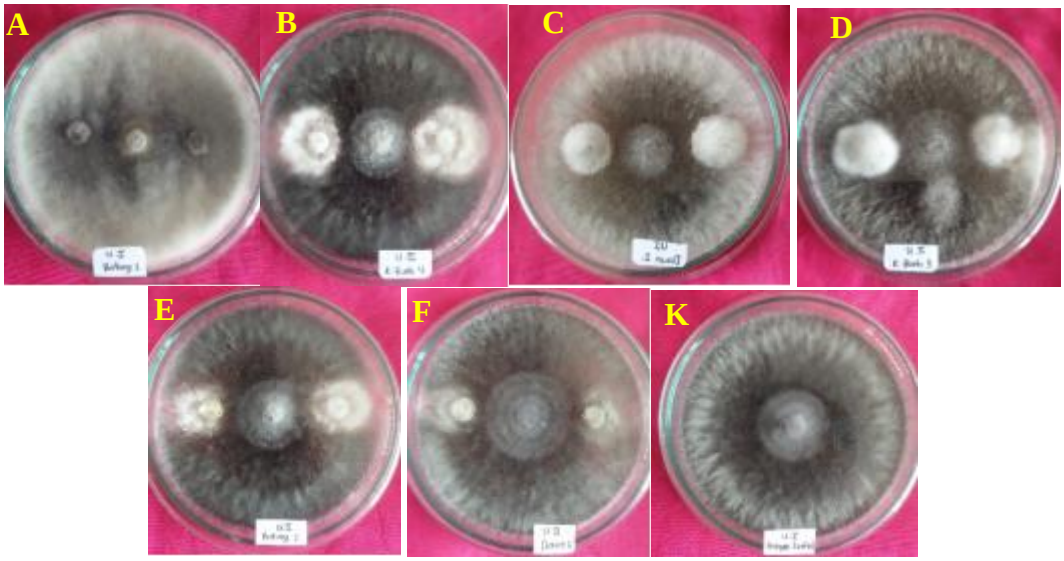

Fig. 3: Inhibition ability test of endophytic fungus on *P. palmivora in vitro,* A =*Lasiodiplodia euphorbicola*, B = *Diaporthe tectonae*, C = *Colletotrichum siamense*, D = *Aspergillus flavus*, E = *Candida parapsilogis*, F = *Aspergillus sydowii*, and K = control (*P. palmivora*) (7 days after inoculation)

Table 1. Percentage of fruit infected by *P. palmivora* each endophytic fungus treatment.

Treatment	Percentage of fruit infected	Notation*
A = control (without antagonistic without pathogen)	100	D
B = *Lasiodiplodia euphorbicola*	0	A
C = *Diaporthe tectonae*	25	B
D = *Colletotrichum siamense*	35	C
E = *Aspergillus flavus*	100	D
F = *Candida parapsilogis*	100	D
G = *Aspergillus sydowii*	100	D
H = control (without antagonistic with pathogen)	100	D

*The same letter in the same column showed no significant difference in the LSD 5%. Data were analyzed after transformed into the form Vx + 0.5.

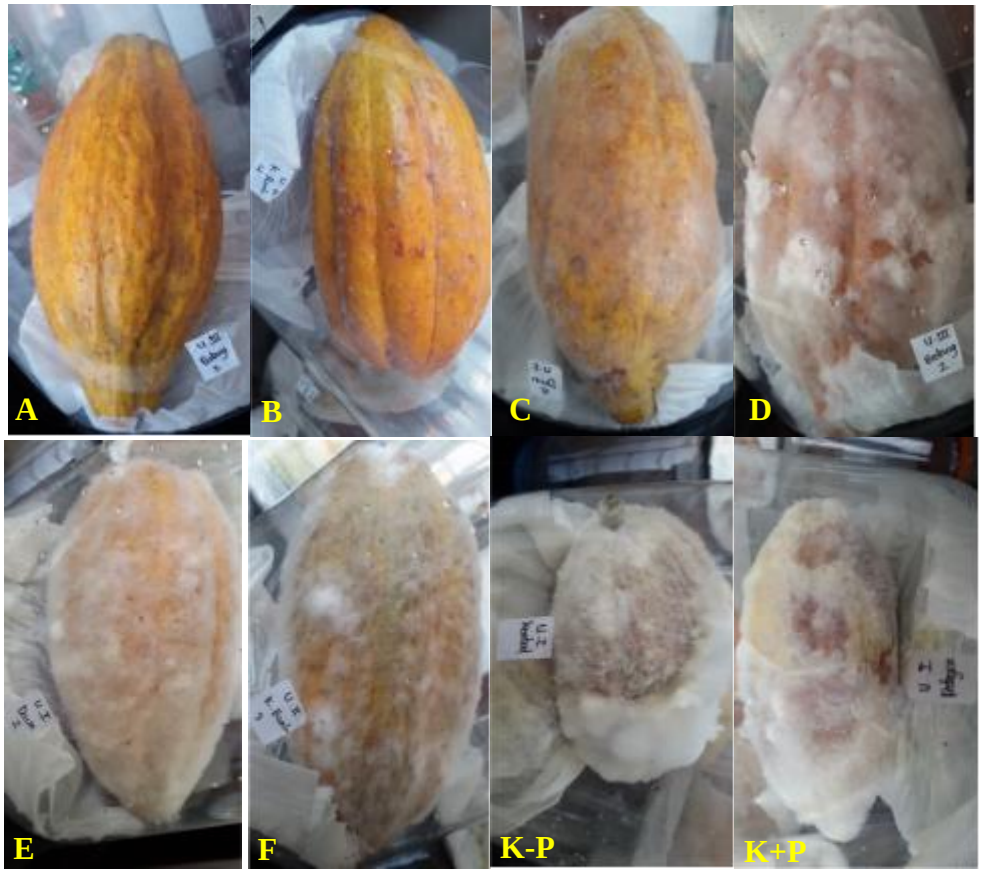

Fig. 4: Antagonistic test in vivo, A = *Lasiodiplodia euphorbicola*, B = *Diaporthe tectonae*, C = *Colletotrichum siamense*, D = *Aspergillus flavus*, E = *Candida parapsilogis*, F = *Aspergillus sydowii*, K-P = control without *P. palmivora*, and K+P = control with *P. plamivora* (7 days after inoculation)

Antagonistic test in the field

The success of the inhibition ability test *in vivo*, followed by success in the field test by using a single type of antagonist, namely *L. euphorbicola*. Seem increasingly concentrated suspension of spore are getting better at pressing *P. palmivora* on cacao black pod. With a spore suspension concentration of the Petri dish 150 ml of sugar solution plus the percentage of infected fruit reached 15%, followed by the concentration of sugar solution of 200 ml and 250 ml together with the percentage of infected fruit 20%, then followed by the addition of sugar solution of 500 ml and 1000 ml with the percentage of infected fruit respectively by 25% and 30% (Table 2, Fig. 5).

Table 2. Disease incidence of *P. palmivora* on fruit in the field.

Treatment	Number of black pod rot (repeats)					Average (%)	Fruit that can be saved
	1	2	3	4	5		
Control	75	75	100	100	100	90 a*	
L. euphorbicola (150 ml)	50	0	0	25	0	15 b	75
L. euphorbicola (200 ml)	0	25	50	25	0	20 b	70
L. euphorbicola (250 ml)	0	50	25	25	0	20 b	70
L. euphorbicola (500 ml)	75	0	25	0	25	25 b	65
L. euphorbicola (1000 ml)	50	0	25	25	50	30 b	60

*****The same letter in the same column showed no significant difference in the level of LSD 5%.

Fig. 5: Endophytic fungus treatment effect against *P. palmivora*, A = Control (without treatment), B = *L. euphorbicola* (150 ml), C = *L. euphorbicola* (200 ml), D = *L. euphorbicola* (250 ml), E = *L. euphorbicola* (500 ml) and F = *L. euphorbicola* (1000 ml) age of 21 days after inoculation.

Discussion

Lasiodiplodia euphorbicola was isolated, which is one cause of the die back disease on the grapevine plants. These pathogens secrete metabolite (-) - mellein, (3R, 4R) - (-) - and (3R, 4S) - (-) - 4-hydroxymellein and tyrosol. Activities phytotoxic metabolites produced also discussed related symptoms these pathogens that cause disease in grapevines (Cimmino et al., 2017). Results showed that *L. euphorbicola* showed inhibition against *P. palmivora* best. The fungus *Diaporthe tectonae* was isolated from soil in Jeon-ju Korea. The isolation is characterized by morphological and phylogenetic analysis using a set of data that is combined with the internal transcribed spacer, β-tubulin, and long sequences of 1-α factor that indicates similar to the strain *D. tectonae* (Park et al., 2017). *Candida parapsilogis* is a fungus that cause disease in humans is able to colonize on the skin, proliferation in a solution containing the sugar and attached to a plastic (Asbeck et al., 2009). *Aspergillus sydowii* been isolated from marine sponges (*Spongia obsura*) cause of illness in the growth of rock in the Caribbean sea (Ein-Gil et al., 2009), while *A. flavus* is a major producer of known aflatoxin human carcinogen (Rodrigues et al., 2007).

Endophytic fungi (*L. euphorbicola*) can suppress the development of fungus *P. palmivora* in the field. The larger the solvent in the form of sugar solution (10%) given the smaller pieces that can be saved, as evidenced by a solution of 150 ml of fruit sugars that can be saved by 75%, and vice versa. The magnitude of the attack of pathogens in the field depends on the source of inoculum. The bigger and near the source of inoculum from healthy fruit, the greater the chance of infection. The reality on the ground that the source of inoculum evenly and planted cultivars are susceptible to *P. palmivora*.

Conclusion

Based on the results of the present study, it can be summarized as follows: fungal antagonists identified based on the sequences of DNA were *Lasiodiplodia euphorbicola*, *Diaporthe tectonae*, *Colletotrichum siamense*, *Aspergillus flavus*, *Candida parapsilogis*, and *Aspergillus sydowii*. Meanwhile, results of inhibition are best shown by the endophytic fungi *L. euphorbicola*. Field trials showed the higher the concentration the better the power spore suspension suppress fungal antagonists against *P. palmivora*. The best concentration that colonies of the Petri dish was dissolved in 150 ml of sugar solution (10%)..

Conflict of interest statement

Authors declare that they have no conflict of interest.

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