

The Potency of Endofit Fungi in Cocoa as Biological Agent to Control Cocoa Pod Disease Caused by *Phytophthora Palmivora* (Butler) Butler

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Abstract. Cocoa pod disease caused by the fungus *Phytophthora palmivora* has resulted in loss of cacao in Indonesia, especially in Bali. So far the disease control strategy is not fully understood. So there is a need to find an alternative by using endophytic fungi associated with cocoa plant. Endophytic fungi are needed to be explored in all parts of the cocoa plant such as stems, leaves and husks. The prevalence of fungal endophyte was determined by the size of its domination on the surface tissue for protection against pathogen. The exploration of the endophytic fungi benefits is aimed at finding biological agents that could control of pathogenic *P. palmivora*. The results showed that 15 types of endophyte fungi have been found in the healthy leaves, stem and pod husks, with the prevalence of fungal endophyte originated from healthy leaf *Mecelia sterilia* (hyphae sterile) around 30%, the endophyte fungi originated from the healthy cocoa stem are *Mycelia sterilia*, *Neurospora* spp and *Trichoderma* spp around 25%. While the endophytic fungi originated from healthy skin fruit is *Trichoderma* spp. around 35%. The in vitro test results of endophytic fungi antagonistic against *P. palmivora* indicated that the endophyte fungi originated from the leaf namely *Aspergillus* spp was obtained at $80 \pm 2\%$, *A. niger* $90 \pm 2\%$, *A. flavus* 100%, and *Trichoderma* spp. $90 \pm 1.5\%$, the endophytic fungus originated from rods namely *Neurospora* spp. was $95 \pm 2\%$, and *Trichoderma* spp. was $90 \pm 2\%$. While the endophytic originated from rind namely *Neurospora* spp. was $95 \pm 1.5\%$ and *Trichoderma* spp. was $80 \pm 2\%$. The results of in vivo test of antagonistic endophytic fungi against *P. palmivora* showed that all of endophytic fungi (*Aspergillus* sp., *A. niger*, *A. flavus*, *Neurospora* sp., and *Trichoderma* sp.) have a significant effect in suppressing the growth of mycelium *P. palmivora*.

Keywords: Endophytic Fungus, *Phytophthora Palmivora*, Inhibition, Prevalence, Biological Agents

I INTRODUCTION

Pod rot is important disease in cocoa cultivation in Indonesia recently, and also in some cocoa producer countries [1]. This disease has average about 20-30% per year on damaging cocoa plant all over the world. In some cases as happen in Samoa America, the cocoa did not planted commercially because of this disease [2]. Cocoa was planted around 532.000 ha in Indonesia on the year of 1999. More than 70% cocoa farmers are the farmer in Indonesia. Indonesia is the top third cocoa exporting countries in the world, produce around 335.000 ton per year, with value 294 million US dollars [3].

Indonesia is the biggest archipelago in the world with has 17.000 islands (6000 has inhabitant). Indonesia is tropical country with various climate and humidity from highland to the lowland. Most of the areas are lowland, while the biggest island has a mountain. Tropical climate with rainy season and high humidity in many areas, some *Phytophthora* diseases caused a significant damage and uncontrollable. *Phytophthora* spp. causes disease on the agricultural plants, horticultural and industrial in Indonesia. At least 11 species *Phytophthora* has been reported as they resulted in economically yield loss in Indonesia. *Phytophthora palmivora* has been identified to

cause an important disease from economical point of view compared to other *Phytophthora* species in Indonesia. *Phytophthora palmivora* infected more than 138 plants species, with caused average loss around 25-50% on cocoa plant, while *P. capsici* caused 52% of decreasing yield on pepper plant. This disease infected many plants but the damage still not be able to be counted, direct damage through fruit infection is showed by the black rotten. Recently, a set of fruits from young fruit to mature one is very sensitive to the infection. [3].

Endophytic fungi which have been isolated from the tissue of healthy give an new hope for biocontrol of cocoa pod disease. The study from Mejia *et al.* (2008) showed that 40% (21/52) of isolated endophytic fungi were able to control *P. palmivora* [4]. One of the isolated fungi which has antagonist characteristic through a simple competition mechanism is *Trichoderma* sp.

II RESEARCH METHOD

Endophyte Isolation

Endophytic fungi used in this study were collected from cocoa planted in Tabanan. The survey of cocoa leaf and fruit was done in four different locations at cocoa plantation centre in Tabanan regency. The obtained leaf and fruit were washed by flowing water with following

methods: 32 pieces of leaf with size 4 mm², the shoot and the flower were cut from the middle of each, the surface was sterilized in 0,525% sodium hypochlorite for 3 minute, and 70% ethanol for 2 minute; and washed by sterile water for 1 minute; and then put in the PDA media (containing antibiotic livoploxacyne 0,1% (w/v). The grown fungi from the piece of leaf were transferred to the tube containing PDA for storage and classified through morphospecies. In order to isolate the endophyte from rod and fruit, those parts were washed by flowing water and then divided into 8 pieces. Fruit were cut into 16 cubical form (8 mm³), 8 from exocarp and 8 from mesocarp. The surfaces were sterilized and storage with similar procedure as isolation from the leaf.

Identification of Endophytic Fungi

The storage endophytic fungi were grown on petri disc containing PDA and repeated 5 times. The cultures were incubated at dark room and temperature $\pm 27^{\circ}\text{C}$. The isolate was identified macroscopically after 3 days of culture periode by observe of the colony colour, growth rate, and microscopically identification was done to investigate septa on the hyphae, the spora form/conidia and the sporangiophore. Fungi identification was done by using some reference books of Samson *et al.*, 1981; Pitt and Hocking, 1997; Barnett and Hunter, 1998; Indrawati *et al.*, 1999 [5][6][7][8].

Prevalence of Endophytic Fungi

Determination of endophytic fungi prevalence were done based on frequency of endophytic fungi isolate were found (eight pieces from leaf, rod, flower and fruit) per petri disc, divided by all founded isolates timed by 100%. The number of isolate prevalence will be determining the dominancy of the endophytic fungi on the healthy cocoa plant.

Inhibition Assay of Endophytic Fungi to the Pathogen

The ability of each endophytic fungi to inhibit the growth of pathogenic *P. palmivora* was tested by dual culture technique (one pathogen colony in the middle and two endophytic fungi next to the pathogen, side by side). Their inhibition can be calculated as follow [9][10]:

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

A = colony diameter *P. palmivora* in the single culture (mm)

B = colony diameter *P. palmivora* in the dual culture (mm).

In Vivo Antagonistic Assay

In vivo antagonistic assay of endophytic fungi was done by impaling the fresh fruit by small splenden needle for 20 times, and then covered by the spore of antagonist fungi (spore from one petri dish was dissolved in 250 ml sterile aquadest), then dyed into the spore of pathogenic fungi (*P. palmivora*). The assays were setting as follows:

A = control (without covering by antagonist)

B = Antagonist treatment 1 (spore suspension 5×10^7)

C = Antagonist treatment 2 (spore suspension 5×10^7)

D = Antagonist treatment 3 (spore suspension 5×10^7)

E = Antagonist treatment 4 (spore suspension 5×10^7)

F = Antagonist treatment 5 (spore suspension 5×10^7)

All of the treatment was repeated 5 times. Experiment was designed by random group design, and after variance analysis by ANOVA following by significance test at level of 5%. The infection parameters were measured by counting the number of infected impale divide by all impale (20 times) timed by 100%.

III RESULTS AND ANALYSIS

Endophytic Fungi

The results showed that endophytic fungi obtained from cocoa plant namely the endophyte from leaf were 7 isolates of *Micelia sterilia* with, 2 isolates of *Aspergillus* spp., 3 isolates of *Aspergillus niger*, 1 isolate of *Aspergillus flavus*, 1 isolate of *Fusarium* sp., 1 isolate of *Mucor* sp., 5 isolates of *Trichoderma* spp. and 1 isolate of *Verticillium* sp. Endophyte from the rod were 5 isolates of *Micelia sterilia*, 1 isolate of *Botryoderma* sp. 1 isolate of *Dactylium* sp. 2 isolates of *Fusarium* sp., 1 isolate of *Oidium* sp. 5 isolates of *Neurospora* spp. and 5 isolates of *Trichoderma* spp. The endophyte obtained from the skin fruit were 3 isolates of *Micelia sterilia*, 1 isolate of *Cylindrocarpon* sp., 2 isolates of *Fusarium* sp. 1 isolate of *Mortierella* sp. 5 isolates of *Neurospora* spp., 1 isolate of *Septocylindrium* sp. and 7 isolates of *Trichoderma* spp. as showed in Table 1 and Fig. 1.

Endophytic fungi normally exist without any symptoms (asymptomatically) in the tissue of the host plant and they have strong attachment to their host. There are two main reasons of thus attachment i.e. first, they are growing indicated that endophyte is able to found in all kind of plant with high abundance and vary. Most of this endophyte are found in internal infection site at leaf, root, rod and skin then transmitted horizontally through the spore. Secondly, the endophyte can produce mycotoxin and convert the physiology and morphology of the host plant. Mycotoxin from endophyte has an advantage to the host plant as acquired plant defenses to face herbivore insect and grass host [11].

Endophyte from leaf and rod of Endofit yang berasal dari daun dan *Hevea brasiliensis* which frequent to found are the genus of *Penicillium*, *Pestalotiopsis* and *Trichoderma* [12]. *Aspergillus* and *Fusarium* produced bioactive compound from the host which contain insecticide activity, cytotoxic and anticancer [13]. The study from Amin *et al.* (2014) have found 6 genus of endophytic fungi in VSD M.05 resistant cocoa plant, namely *Curvularia* sp., *Fusarium* sp., *Geotrichum* sp., *Aspergillus* sp., *Gliocladium* sp., and *Colletotrichum* sp., and another four were not be able to identified [14].

Prevalence of Endophytic Fungi

This study showed that prevalence of endophytic fungi originated from healthy leaf is *Mycelia sterilia* (sterile hypha) around 30%, in endophyte from healthy cocoa rod are *Mycelia sterilia*, *Neurospora* spp. *Trichoderma* spp. around 25% of each. While in the endophyte from the fruit skin is *Trichoderma* spp. 35%

(Table 1). *Trichoderma* spp., are the dominant genus in south part of China (4 isolates, 23,5%). It has been reported that endophytic fungi can produce antitumor or antifungi activity. The fungi which has been isolated from

the skin fruit of medicinal plant including *Pasecilomyces* sp., *Cephalosporium* sp., *Mortierella* sp., *Mucor* sp., *Trichoderma* sp., and *Cladosporium* sp. [15].

TABLE 1.
THE ENDOPHYTIC FUNGI PREVALENCE ON THE LEAF, ROD AND FRUIT OF THE HEALTHY COCOA PLANT.

Fungi	Endophyte from the leaf	Endophyte from the rod	Endophyte from the fruit skin
<i>Micelia sterilia</i>	6 (30%)*	5 (25%)	3 (15%)
<i>Aspergillus</i> spp.	2 (10%)	-	-
<i>Aspergillus niger</i>	3 (15%)	-	-
<i>Aspergillus flavus</i>	1 (5%)	-	-
<i>Botryoderma</i> sp.	-	1 (5%)	-
<i>Cylindrocarpon</i> sp.	-	-	1 (5%)
<i>Dactylium</i> sp.	-	1 (5%)	-
<i>Fusarium</i> sp.	1 (5%)	2 (10%)	2 (10%)
<i>Oidium</i> sp.	-	1 (5%)	-
<i>Mortierella</i> sp.	-	-	1 (5%)
<i>Mucor</i> sp.	1 (5%)	-	-
<i>Neurospora</i> spp	-	5 (25%)	5 (25%)
<i>Septocylindrium</i>	-	-	1 (5)
<i>Trichoderma</i> spp.	5 (25%)	5 (25%)	7 (35%)
<i>Verticillium</i> sp	1 (5%)	-	-
Total	20	20	20

*Note: the percentage is prevalence (frequency of isolates)

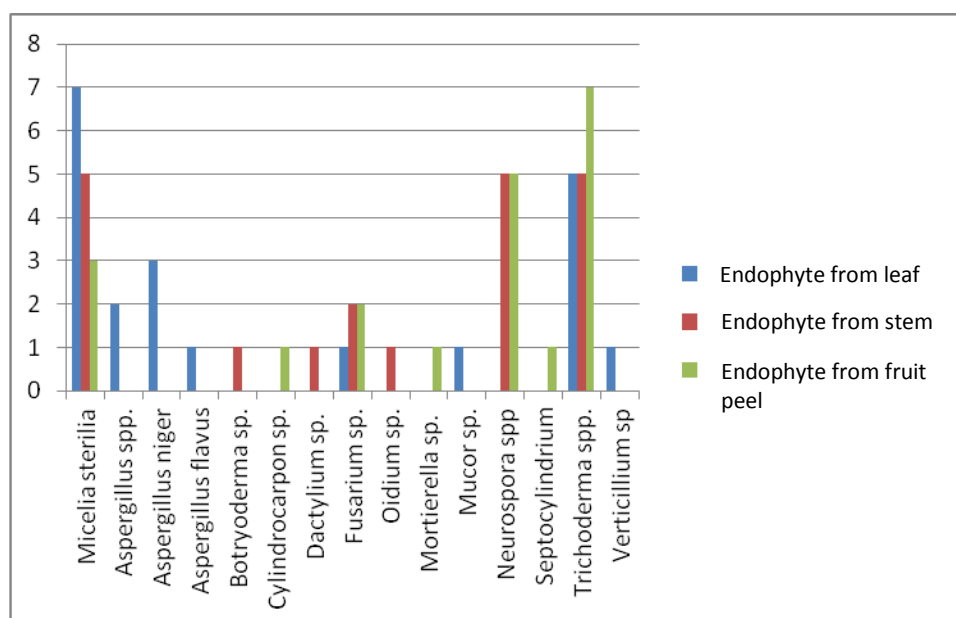


Fig. 1. Endophytic fungi originated from leaf, rod and fruit skin of cocoa plant.

The Results of Antagonistic Assay Endo-phytic Fungi on *Phytophthora palmivora*

The result of this study showed that its obtained around 80±2% of *Aspergillus* spp., 90±2% of *A. niger*, 100% of *A. flavus* and 90±1,5% of *Trichoderma* spp in endophyte from leaf. 95±2% of *Neurospora* spp. and 90±2% of *Trichoderma* spp. are obtained from endophyte which originated from rod. While 95±1,5% of *Neurospora* spp. and 80±2% of *Trichoderma* spp. from endophyte originated from the fruit skin (Table 2, Fig. 2).

Aspergillus flavus and *A. terreus* which growing internally are seed transmitted fungi that can inhibit the growth of pathogenic *Rhizoctonia solani* Khun, and then it's useful for the control of seed transmitted diseases which infected several important plant [16]. Most endophyte which is found in cocoa rod are the genus that familiar known as soil fungi (*Clonostachys* and *Trichoderma*) [4].

TABLE 2.
INHIBITORY ASSAY OF ENDOPHYTIC FUNGI TO *P. PALMIVORA*

Fungi	Endophyte from leaf	Endophyte from rod	Endophyte from fruit skin
<i>Micelia sterilia</i>	-	-	-
<i>Aspergillus</i> spp.	80±2%	-	-
<i>Aspergillus niger</i>	90±2%	-	-
<i>Aspergillus flavus</i>	100%	-	-
<i>Botryoderma</i> sp.	-	-	-
<i>Cylindrocarpon</i> sp.	-	-	-
<i>Dactylium</i> sp.	-	-	-
<i>Fusarium</i> sp.	-	-	-
<i>Oidium</i> sp.	-	-	-
<i>Mortierella</i> sp.	-	-	-
<i>Mucor</i> sp.	-	-	-
<i>Neurospora</i> spp	-	95±2%	95±1,5%
<i>Septocylindrium</i>	-	-	-
<i>Trichoderma</i> spp.	90±1,5%	90±2%	80±2%
<i>Verticillium</i> sp	-	-	-

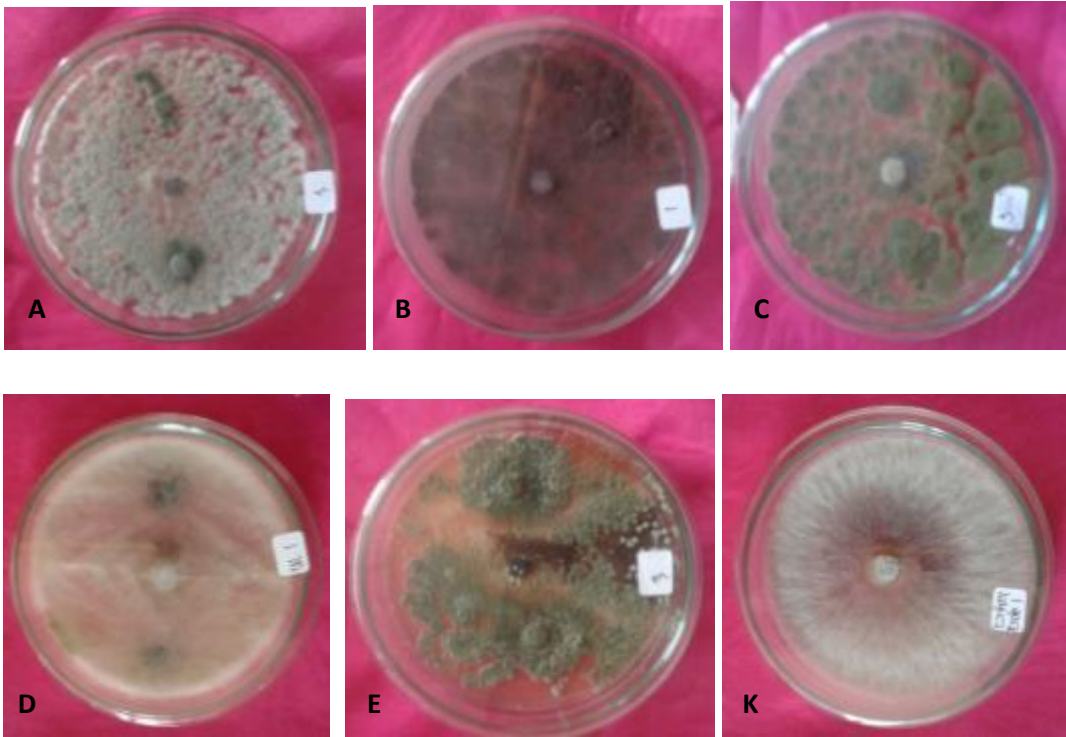


Fig. 2. Antagonistic assay of endophytic fungi to *P. palmivora*, (A) *Aspergillus* sp., (B) *Aspergillus niger*, (C) *Aspergillus flavus*, (D) *Neurospora* sp., (E) *Trichoderma* sp., and (K) Control (*Phytophthora palmivora*) 7 day after incubation

Trichoderma sp. also found as endophtic fungus which is able to inhibit *Crinipellis pernicioso* (Stahel) Singer, the main cause of *Witches Broom* disease on

cocoa plant [17]. *Trichoderma*, has potency as a biological agent to control *Phytophthora* spp., moreover

Fusarium and *Verticillium* are antagonistic to some pathogenic fungi [18].

In Vivo Antagonistic Assay

The *in vivo* antagonistic assay showed that all five biological agents have a significant different on inhibition of the *P. palmivora* growth. The five biological agents that tested on the fresh fruit i.e. *Aspergillus* sp., *A. niger*, *A. flavus*, *Neurosporas* sp., *Trichoderma* sp. (Fig. 3).

Among endophytic fungi founded on *Hevea brasiliensis*, *Trichoderma* is the most often been found and isolated [12], and is able to inhibit the growth of *P. palmivora* either by competition or antibiosis mechanism. The percentage of infection are significantly different in all treatment compared to the control (Fig. 3, Table 3). Antagonistic treatment using *Aspergillus* sp. has percentage infection around 10%, while the other (*A. niger*, *A. flavus*, *Neurospora* sp. and *Trichoderma* sp.) has percentage infection around 5% each.

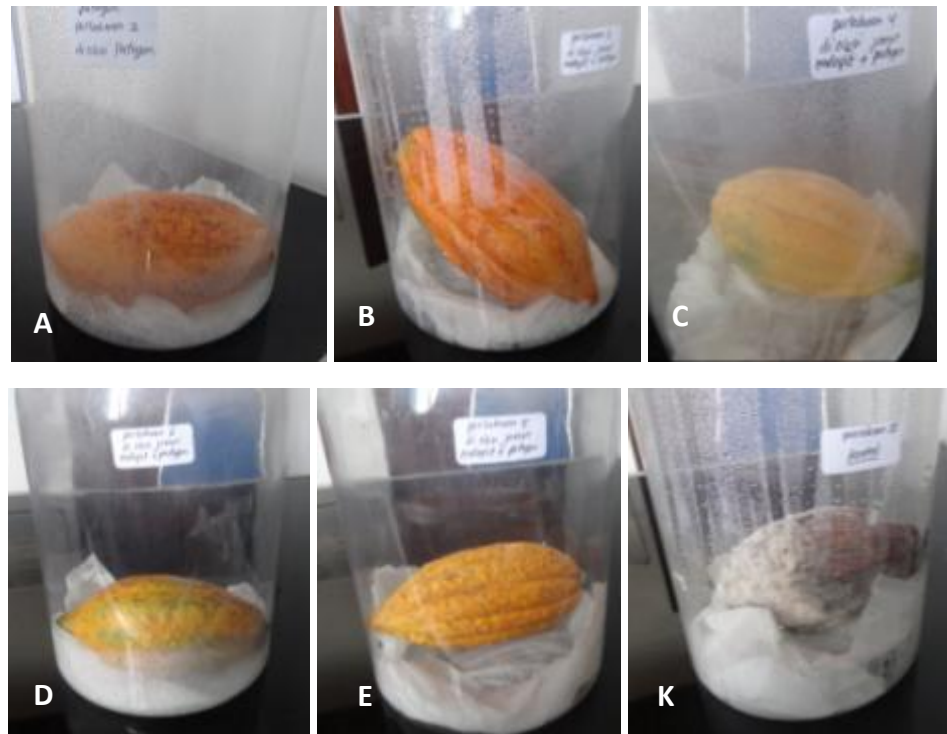


Fig. 3. The result of *in vivo* antagonistic assay on fresh fruit (A) *Aspergillus* sp., (B) *Aspergillus niger*, (C) *Aspergillus flavus*, (D) *Neurospora* sp., (E) *Trichoderma* sp. and (K) Control 7 days after incubation

TABLE 3.
THE INFECTION PERCENTAGE OF *P. PALMIVORA* ON THE FRUIT IN EACH TREATMENT.

No.	Tretment	Percentage of Infection
1	Control	100 ± 0 a **
2.	Treatment with <i>Aspergillus</i> sp.	10 ± 0,2 b
3.	Treatment with <i>Aspergillus niger</i>	5 ± 0,1 b
4.	Treatment with <i>Aspergillus flavus</i>	5 ± 0,3 b
5.	Treatment with <i>Neurosporas</i> sp.	5 ± 0,2 b
6.	Treatment with <i>Trichoderma</i> sp.	5 ± 0,1 b

** Significant different at BNT 1%.

IV CONCLUSION

Endophytic fungi which have been found on the leaf, rod and fruit skin of healthy cocoa are 15 species, with prevalence of endophytic fungi from healthy leaf are *Mecelia sterilia* (sterile hifa) around 30%, on the endophyte from healthy rod it's found mycelia sterilia, *Neurospora* spp. and *Trichoderma* spp. around 25% of each. While at the endophyte originated from fruit skin, it's found *Trichoderma* spp. around 35%. The *in vitro* antagonistic assay results of endophytic fungi to *P.*

palmivora showed that at the endophyte from leaf its obtained *Aspergillus* spp. around 80±2%, *A. niger* 90±2%, *A. flavus* 100%, and *Trichoderma* spp. around 90±1,5%. On the endophyte from rod, it's obtained 95±2% of *Neurospora* spp. and 90±2% of *Trichoderma* spp. While endophyte from the fruit skin, its obtained 95±1,5% of *Neurospora* spp. and 80±2% of *Trichoderma* spp. The result of *in vivo* antagonistic assay showed that all of endophytic fungi (*Aspergillus* sp., *A. niger*, *A. flavus*, *Neurospora* sp., dan *Trichoderma* sp.) have a significant effect on inhibition the mycelium growth of *P. palmivora*.

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