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Endophytic fungi as potential biocontrol agents of *Phytophthora palmivora* in the cocoa plant

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Abstract. *Simamora AV, Hahuly MV, Henuk JBD. 2021. Endophytic fungi as potential biocontrol agents of* Phytophthora palmivora *in the cocoa plant. Biodiversitas 22: 2601-2609.* In Indonesia, the cocoa tree is one of the essential cultivation crops for farmers. Despite the importance of cocoa cultivation in Indonesia's economy, the productivity of this crop has declined. Cocoa black pod disease caused by *Phytophthora palmivora* (Butl.) is one of the most severe diseases affecting this crop worldwide, with average annual losses above 40%. Instead of using manufactured chemicals, biological control is an effective and eco-friendly alternative control measure against plant pathogens. This work aimed to assess the potential of endophytic fungi isolated from healthy cocoa pods to control *Phytophthora palmivora in vitro* and *in vivo*. Endophytic fungal isolates were classified based on the morphological characteristics of their cultures and reproductive structures. All isolates found were tested to inhibit *P. palmivora* in the dual culture method, and the best 10 isolates were continued for detached pod assay. Then, the best five isolates (*Aspergillus*4, *Aspergillus*5, *Aspergillus*6, *Fusarium*6, *Ramichloridium* sp.) were evaluated for their capability to reduce *P. palmivora* in cocoa seedlings and in the field plants. *Aspergillus*, *Fusarium*, and *Ramichloridium* showed maximum activity against *P. palmivora* in dual culture, pod, and seedling assays. Nevertheless, when all these five isolates were applied in the field, they did not suppress the disease development.

Keywords: *Aspergillus*, cocoa, endophytic fungi, *Fusarium*, *Ramichloridium*, *Phytophthora palmivora*

INTRODUCTION

Cocoa is one of the most attractive plants for farmers around the equator due to the specific climate and inevitability imperative of profits over in the long term. Indonesia's equatorial climate, with its soil fertility, offers the best geographic conditions for the cultivation of cocoa trees. Witjaksono and Asmin (2016) stated that over 70% of Indonesian cocoa production is focused on Sulawesi Island. Outside Sulawesi, the cocoa production regions are located in North Sumatra, West Java, and Papua, Bali, Flores, and other islands. Cocoa trade offers the primary source of salaries for more than 1,400,000 smallholder growers and their households in Indonesia.

Indonesia's export value of processed cocoa products during January-June 2020 amounted to 549 million USD, an increase of 5.13 percent compared to the same period in 2019. Despite the contribution of cocoa farming in the Indonesian economy, cocoa production has gradually declined from 410.000 tonnes in 2012/2013 to 290.000 tons in 2019/2019 and to 200.000 tonnes in 2020, affecting this country downgraded from the 3rd to 5th and then to the 6th highest cocoa producing country in the world (Sella and Fardah 2020; Praseptiangga et al. 2020).

The decline in cocoa productivity can be attributed to low farm management practices, old trees, and the incidence of pests and diseases (Wardhany and Adzim 2018). One of the most harmful diseases for cocoa cultivation worldwide, including in Indonesia, is black pod rot disease caused by *Phytophthora palmivora*. This pathogen attack on cocoa plantations has caused yield losses of 20%–40% worldwide, and the yield loss increased, especially in areas with high rainfall and humidity (Villamizar-Gallardo et al. 2017; Perrine-Walker 2020). In Indonesia, losses due to this disease can reach up to 90%, especially during the rainy season (Gassa et al. 2016). Besides pod, *P. palmivora* also infect bark, flower cushions, stem, and branch causing cankers. *Phytophthora* canker's importance is perhaps underrated, as cankers diminish plant vigor and fruit-bearing capability, therefore decreasing harvest. Stem cankers may also develop a significant source of inoculum for pod rot. Likewise, in wet conditions, *P. palmivora* produces seedling and leaf blight (Guest 2007). Seedling blight often develops wilting of stems and leaves, defoliation, and finally death within one week after inoculation (Nur'Aini et al. 2016), and the disease can cause total loss if not managed (Peter and Chandramohanan 2014).

Currently, incorporated pest and disease control methods such as phytosanitation, pruning, chemical fungicide, resistant cultivars, and biological agents are recommended to suppress pod rot disease. Still, this disease remains a problem worldwide (Hanada et al. 2009; Rubiyo and Amaria 2013). Conventional chemical control of black pod rot disease can be costly, unproductive, and risky for both ecological and human safety. As part of integrated pest management, biological control has been recommended as the best natural, environmentally friendly remedy (De Silva et al. 2019).

One of the promising new agents in the biological control of plant diseases is fungal endophytes. Fungal endophytes are defined as fungi residing in plant tissue and

do not show disease symptoms (Chitnis et al. 2020). These endophytic fungi protect their hosts from harmful organisms and unfavorable environments by producing bioactive secondary metabolites directly and indirectly (Fadiji and Babalola 2020a). According to Nur Amin (2013), Nur Amin et al. (2014 and 2015), endophytic fungi protect their host plant against pest and pathogen, i.e., root-knot nematode *Meloidogyne* spp., cocoa pod borer *Conopomorpha cramerella*, and *Oncobasidium theobromae*, the causal agent of vascular streak dieback disease on the cocoa seedling. Similarly, Mejía et al. 2008 reported that endophytic fungi could control three primary diseases of cocoa, i.e. black pod rot disease caused by *Phytophthora* spp.; frosty pod disease caused by *Moniliophthora roreri*; and witches broom disease caused by *Crinipellis perniciosa*. In some cases, fungal endophytes inhibited the growth of cocoa pathogens both in laboratory and field studies (Hanada et al. 2009, 2010). All results emphasized that fungal endophytes are important as a contemporary biological control agent for controlling pathogenic diseases of cocoa. This current work emphasis on (i) assessing and identifying endophytic fungi isolated from healthy cocoa pods collected from small scale cocoa farmings in three sub-districts of Sikka District, East Nusa Tenggara (ENT), Indonesia, (ii) evaluating the antagonistic effect of selected endophytes against *Phytophthora palmivora in vitro* and *in vivo*.

MATERIALS AND METHODS

Isolation of endophytic fungi

Three-month-old of healthy cocoa pods were collected and kept in a sterile plastic container. All pods were brought to the laboratory benches for processing within 48 h after harvesting. Exterior cleansing was carried out according to Myrchiang et al. 2014. The pods were first cleaned by running tap water to eliminate rubbish usually abiding on pods' surface. After that, all pods were disinfected in 70% ethanol for 1-3 minutes followed by 4% sodium hypochlorite (NaClO) solution for 3-4 minutes, then rinsed with 70% ethanol for 2-5 seconds and lastly washed twice with sterile distilled water for one minute to eradicate all the disinfectants. The disinfected pods were sliced into small pieces of 5 mm x 5 mm with a sterilized blade. Each sample was blot dried out under sterile conditions. Four to six slices of the cutting pod were put in Petri dishes containing potato dextrose agar (PDA) medium with streptomycin (100 mg L^{-1}). The composition of PDA was 200 g L^{-1}diced potatoes, 20 g L^{-1}dextroses, and 20 g L^{-1}agar. The medium was prepared by adding the potato infusion (boiled filtrate of diced potatoes) along with dextrose and agar and sterilized by autoclaving at 121˚C, 15 lb pressure for 15 min. The inoculation was carried under a laminar airflow chamber, and after the inoculation, the dishes were labeled accordingly and incubated at laboratory benches at 28±2˚C.

The Petri dishes were observed daily to assess the fungal growth. After several days of incubation, purity of each isolate was assessed by examining the colony morphology. All the isolates were purified and pure culture was maintained in PDA slant tubes.

Characterization of endophytic fungi

All the endophyte isolates were characterized based on their macroscopic and microscopic features and their reproductive structures under a microscope (10X and 40X) using routine procedures (Barnett and Hunter 1998; Watanabe 2010). Macroscopic observation was based on the fungal colony's characteristics on agar medium such as colony diameter, color, texture, reverse side, margins, and pigment production (Olokkaran et al. 2019). Microscopic features were monitored for the presence or absence of conidia, conidia shape and size, conidial arrangement, conidiophores, hyphae (septate or non-septate). Identification was performed by wet mount technique in which the fungal colonies were stained using lactophenol cotton blue.

Subculturing of *Phytophthora palmivora*

Phytophthora palmivora isolate was obtained by Plant Disease Laboratory, Universitas Nusa Cendana. *Phytophthora palmivora* was isolated from diseased cocoa pods (with symptoms of blackish-brown spots). The isolation procedure was as follows: diseased cocoa pods were surface sterilized using 70% ethanol. Then, the outer skin of cocoa pods was removed using a sterile knife. At the edge between diseased and healthy tissue, 0.5 × 0.5 cm^2 of pulp was taken, then mixed in 2% water agar (WA) media. The culture was then incubated for three days until hyphae grew on the surface of the tissue. The growing hyphae were transferred to PDA media, incubated at room temperature, then purified and identified.

Evaluation of antagonistic effects of endophytic fungi

The antagonistic activity of fungal endophytes was evaluated based on the technique of Mejía et al. 2008. The types of interaction between endophytes and *P. palmivora* were evaluated 60 h after they were incubated in the Petri dishes. The interactions observed were antibiosis, competition, and mycoparasitism. All endophytic fungi isolated were tested for their antagonism against *Phytophthora palmivora* by using dual culture technique as explained by Myrchiang et al. 2014 with minor modifications. The steps were as follows: (i) 5 mm diameter of mycelial plugs of fungal endophytes and *P. palmivora* were plated on the reverse edge of the PDA plate. (ii) The Petri dishes were set in five replicates. For control, *P. palmivora* was plated with sterile agar plugs. (iii) The Petri dishes were arranged in laboratory benches at 25±2˚C for seven days. (iv) The growth of *P. palmivora* was assessed against all the fungal endophytes. Data on the growth of the *P. palmivora* and fungal endophytes were observed every day. (v) The inhibition percentage of radial growth of *P. palmivora* by endophytes was computed using the formula:

$$\text{Inhibition percentage (\%)} = (R1 - R2)R1^{-1} \times 100$$

Where R1 = radial growth of *P. palmivora* in control Petri dish; R2 = radial growth of *P. palmivora* towards the

antagonist in test Petri dish. The percentage of inhibition was analyzed using analysis of variance (ANOVA) and continued with least significant difference (LSD) test.

Testing the ability of endophytic fungi to reduce *Phytophthora palmivora* lesions on detached cocoa pods

Based on *in vitro* antagonisms test, 10 endophytic fungal isolates were chosen for the detached cocoa pods test. Approximately four-month-old cocoa pods were collected from small-scale cocoa farms in Nebe and Munerana Villages, Sikka, ENT, Indonesia. The day before the pods were collected, the farm was checked and verified to be healthy without any injury. All the collecting pods were brought to the laboratory for further processing. All endophytic fungal spores were collected from seven-day-old cultures plated on PDA. The conidia suspension was prepared by cleansing the cultured dishes with sterile distilled water. The concentration of conidia was adjusted using a hemocytometer 1 x10^6 spores mL^{-1}, 2% tween 20 LC was added to obtain a homogeneous suspension (Hanada et al. 2010), and coconut oil was included as an adhesive (Hanada et al. 2010; Sriwati et al. 2015). The conidia suspension was applied to the entire pod surfaces using a garden sprayer. Sprayed pods were kept in a plastic container at 25±2°C for 24 hours to maintain moisture. Twenty-four h after applying endophytic fungi isolates, pods were infected with *P. palmivora* in this way. The epicarp of each pod was cut at four dots with a distant of 1 cm using a sterilized scalpel. The exposed parts were sealed with 5 mm diameter agar plug taken from 1-week old *P. palmivora* culture. Pods were kept for seven days at 25±2°C in a 40 cm x 30 cm plastic container. The treatments were arranged in a completely randomized design with five replicate pods. One pod inoculated with one fungal endophyte. Observations were made on disease symptoms and spot diameter of *P. palmivora* on the surface of the cocoa pods. The experiment was conducted twice. Data from the two experiments were averaged and analyzed. Spot diameter was analyzed using Anova and followed by LSD test.

Screen-house experiment

Three-month-old healthy cocoa seedlings were collected from small scale farming in Munerana Village, Sikka District, ENT, Indonesia. Seedlings were grown in a 5 kg polybag filled with sterilized field soil and compost (2:1) and were irrigated daily but the above-ground parts were kept waterless. Five chosen fungal endophytes (*Aspergillus*4, *Aspergillus*5, *Aspergillus*6, *Fusarium*6, *Ramichloridium* sp.) were inoculated on cocoa leaves. All five endophytes showed good antagonistic ability to inhibit *P. palmivora* in dual culture and pod assays. The spore suspensions of all endophytic isolates were prepared as described before. Spores were sprayed on all aerial parts of the plant in the late afternoon by a garden sprayer until the whole seedlings were soaked. Uninoculated seedlings were applied with sterilized water. The seedlings were set on benches and arranged in a randomized block design with five replicates. High relative humidity was maintained in the screened house for 48 h following by placing a wet cloth between the seedlings (Mejía et al. 2008).

Two weeks after the endophytes application, the seedlings were inoculated with *P. palmivora*. *Phytophthora palmivora* sporangia were collected from black pod rot disease according to the procedure of Sriwati and Muarif (2012). The concentration of *P. palmivora* sporangia was adjusted to 1 x 10^6 sporangia mL^{-1} using hemocytometer. The sporangia suspension was applied to the seedlings by a garden sprayer. After the application, the plants were protected with a plastic cover to retain moisture for one day (Sriwati et al. 2015). Two weeks after the application of *P. palmivora*, seedlings were monitored for disease symptoms and disease incidence (DI). DI was calculated as follows: DI = ($n.N^{-1}$) x 100%, with n= number of infected seedlings and N= number of observed seedlings. The experiment was conducted twice. The average data from the experiment were used for analysis. Disease incidence was analyzed using Anova and continued with LSD test.

Field trial

Five endophytic isolates formerly assessed under screen house settings were selected for the field trial. The trial was conducted in a cocoa farm Wolomotong Village, Sikka Regency, ENT, where biological agents were never applied. The garden used was owned by a farmer whose cocoa pods were heavily infected with *P. palmivora*. The experimental pods were 3 to 4 months old attached to the tree. The pods used were categorized into four groups of severity: (i) 0% (healthy); (ii) Pod severity >10≤25%; (iii) Pod severity >25<50%; and (iv) Pod severity ≥50%. Inoculation of fungal endophytes on cocoa pods was conducted according to the predetermined category. Different pod categories could be fixed in one tree, but only one endophyte was applied in one tree. Distance between cocoa trees was 1.5-3.0 m. The treatment consisted of five isolates of endophytic fungi (*Aspergillus*4, *Aspergillus*5, *Aspergillus*6, *Fusarium*6, *Ramichloridium* sp.), which were sprayed directly on the cocoa pods, with three replications (three pods). The spore suspensions (1 x 106 mL^{-1}) of all endophytic isolates were prepared as described previously and applied to the pods surface until near overflow, using a garden sprayer. In this method, the amount of suspension sprayed on each pod was 40–50 mL, taking into account the cocoa pod's regular size. The cocoa pods were covered with clear plastic to maintain the humidity, placed 24 h before applying treatments, and took off one day after that, tolerating the trial to be performed under normal field circumstances (Hanada et al. 2009). The healthy pods were inoculated with sterile water. Disease severity was recorded every week for four weeks.

RESULTS AND DISCUSSION

Isolation and characterization of endophytic fungi from cocoa pods

Total of 25 isolates of endophytic fungi were isolated from healthy cocoa pods. All isolates were classified to the genera taxon, but one isolate remains unidentified. The isolates found were seven isolates of *Fusarium*, six isolates

of *Aspergillus*, three isolates of *Gliocladium*, three isolates of *Cylindrocladium*, three isolates of *Mortierella*, one isolate of *Ramichloridium*, and one isolate of *Rhizoctonia*.

Result showed that all fungal isolates were basically belonging to Class 2 endophytes, Ascomycota (96%) and Basidiomycota (4%), but because they only exhibited asexual form during examination, all isolates belonged to the Deuteromycota group or imperfection fungi. Generally, fungal endophytes are grouped into clavicipitaceous endophytes (C-endophytes) and the non clavicipitaceous endophytes (NC-endophytes. These two main groups of endophytic fungi show variation in evolutionary correlation, taxonomy, plant hosts, and ecological roles. The C-endophytes abide in some grasses while NC-endophytes are attained from vascular and non-vascular plant species (Jain and Pundir 2017). NC-endophytes are very widespread, they have been regained from each major ancestry of terrestrial floras and all continental ecosystems, containing agroecosystems and biomes varying from tropics to tundra. NC-endophytes are divided into three functional classes (Class 2, 3 & 4 endophytes) based on host colonization model, transmission methods between host generations, in planta biodiversity ranks, and ecological role (Lugtenberg et al. 2016). The assorted class 2 endophytes include both Ascomycota and a small number of Basidiomycota (Abo Nouh 2019).

In this study, *Fusarium* and *Aspergillus* were the most prevalent genera with 7 (28%) and 6 (24%) isolates found, respectively. The frequency of these genera was inconsistent with findings of other research (Nur Amin et al. 2014), even though the ratios were different because the test plant for sampling was different. Two genera i.e. *Ramichloridium* and *Rhizoctonia* obtained as individual isolates.

Species of the genus *Ramichloridium* show various morphological characteristics and lifestyles, such as plant pathogens, commensals, and saprophytes (Kirschner and Piepenbring 2014), but Zheng et al. (2020) reported that *R. endophyticum* was obtained as an endophyte from an aquatic macrophyte *Potamogeton pectinatus*. Xie et al. (2016) also reported that a Class 2 endophytes, *Ramichloridium cerophilum* isolated from the leaf of *Sonchus oleraceus*, has displayed the ability to improve biomass of Chinese cabbage, a nonmycorrhizal plant. Fitriarni and Kasiamdari (2018) stated that *Rhizoctoni*a was obtained as a fungal endophyte from leave and stem of *Calopogonium mucunoides* and Rashmi et al. (2019) informed that *Rhizoctonia* spp. were isolated as endophytic fungi from several plants such as *Fragaria vesca*, *Brassica napus*, *Taxus mairei*, and *Canarium ovatum*.

In vitro antagonistic assay

All the endophytic fungi tested against *P. palmivora* showed *in vitro* antagonism. The competition was the most common mode of action against *P. palmivora* (Table 1). This assay exhibited that all fungal endophytes examined had an effective antagonism to suppress *P. palmivora*, the cocoa's black rot pod pathogen. All the tested antagonists noticeably inhibited the radial growth of the pathogen. In the control plate (*P. palmivora* minus endophytes), *P. palmivora* grew rapidly and colonized the entire plate within seven days, whereas in dual culture *P. palmivora* takes longer time to develop. The percentage of inhibition of radial growth of *P. palmivora* by all the tested fungal endophytes was significantly different. *Fusarium*6 was recorded to be the best aggressive isolate and constrained the radial development of the *P. palmivora*, followed by *Aspergillus*5, *Ramichloridium* sp., *Fusarium*5, and *Gliocladium*3.

The inhibition of *P. palmivora* by endophytic fungi varied substantially, due to the different mechanisms of each isolate. Fadiji and Babalola (2020a) stated that the mechanism of inhibiting pathogens growth by endophytic fungi can be by direct parasitizing of the pathogens, production of antibiotics, competition for place and food, production of enzymes, and initiation of plant resistance responses. Endophytic fungi showed inhibition against *P. palmivora* by various mechanisms, namely competition (15 isolates = 60%), antibiosis (8 isolates = 32%), and mycoparasites (2 isolates = 8%) (Table 1, Figure 1). In a competition mechanism, the growth of endophytic fungi was faster than that of *P. palmivora* so that all the space was filled with endophytic fungi, and the growth of *P. palmivora* was inhibited (Figures 1.B and 1.C). The antibiosis mechanism can inhibit pathogens by producing antibiotics, enzymes, and toxins (Figures 1.A). In mycoperitism, endophytic fungi destroyed or deformed the cell walls of *P. palmivora* (Figure 1.D).

*Fusarium*4 **A** | *Aspergillus*4 **B** | *Aspergillus*6 **C** | *Gliocladium*2 **D** | *P. palmivora* **E**

Figure 1. Inhibition growth of four endophytic fungi from cocoa pods to *Phytophthora palmivora*. A= mechanism of antibiotic; B and C= mechanism of competition; D= mechanism of mycoparasite. Pictures A-D were taken seven days after inoculation of both endophytic fungi and *P. palmivora* in the Petri dishes, E= control, seven- day old *Phytophthora palmivora*

The mechanism of inhibition of *P. palmivora* by endophytic fungi due to antibiosis, which was characterized by a clear zone around endophytic fungi. The mechanism of antibiosis may include the production antibiotics or secretion of lytic enzymes (Fadiji and Babalola 2020a). Furthermore, Fadiji and Babalola (2020a) stated that metabolites produced by endophytic fungi are categorized into separate groups such as alkaloids, terpenoids, phenols, steroids, and polyketides.

Sufficient data are available that fungal endophytes comprise an essential role in host-plant physiology. They accept food, security, and dissemination from their host, whereas host plants are profited from this symbiosis as well (Fadiji and Babalola 2020a). Endophytes offer shelter to their hosts from destructive organisms and support their hosts to acclimate in varied adverse environments. Methods opted by endophytic fungi for plant growth stimulation are competition, phosphate solubilization, and the production of siderophore, phytohormone, cell wall-lysing enzymes, and antibiotics (Vyas and Bansal 2018; Fadiji and Babalola 2020a).

These *in vitro* results indicated that *Fusarium* spp., *Aspergillus* spp., *Ramichloridium* sp, and *Gliocladium* spp. could inhibit the growth of *P. palmivora*. Other investigations have verified the capability of these endophytic fungi to control pathogens. Gautam et al. (2013) concluded that endophytes *Aspergillus niger* and *A. flavus* isolated from *Cannabis sativa* prevent the growth of two common phytopathogenic fungi, *Colletotrichum gloeosporioides*, and *Curvularia lunata*. Similar result from Wei et al. (2019) also revealed that *Fusarium solani*, a fungal endophyte strain isolated from cotton, exhibits protection against *Verticillium dahliae*, a soilborne pathogen causing verticillium wilt of cotton, one of the most severe diseases of cotton worldwide. *Gliocladium* isolated from the *Eucryphia cordifolia* releases a combination of volatile organic compounds toxic to *Pythium ultimum* and *Verticillium dahliae* (Stinson et al. 2003). *Ramichloridium* sp. obtained from indigenous *Euterpe precatoria*, performed a unique capability to manage anthracnose disease caused by *Colletotrichum gloeosporioides* (Peters et al. 2020).

Testing endophytic fungi ability to reduce *Phytophthora palmivora* lesions on detached cocoa pods

Result showed that all tested isolates suppressed *P. palmivora* infection compared to control (Table 2). The strongest suppression was exhibited by *Aspergillus*6 but insignificantly different with *Aspergillus*4 and *Fusarium*6 isolates. All endophytic fungi use different methods of antagonism against *P. palmivora*. Fungal endophytes protect plants by employing both direct and indirect mechanisms. There are three primary methods by which endophytic fungi possibly develop plant defense to phytopathogens (Fadiji and Babalola 2020a). First, the endophytes decline the pathogens by parasitizing them directly or other antimicrobial complexes, or by producing phytoalexin, and biocidal compounds. Second, endophytes defeat pathogens by confronting space and food, or by supplying enzymes that attack the cell elements of pathogen. Third, endophytes suppress pathogens by secreting antibiotic elements (Myrchiang et al. 2014). The antagonistic effect of *Aspergillus* spp., *Ramichloridium* sp., *Gliocladium* sp., and *Fusarium* sp. isolates in this study may be due to the combination of all three mechanisms (Myrchiang et al. 2014; Fadiji and Babalola 2020a).

The capability of endophytic fungi to reduce the growth of pathogens was confirmed by previous studies. Hanada et al. (2010) concluded that the use of endophytic fungi against *P. palmivora* could suppress the development of pathogens in cocoa pods. Furthermore, Sreeja et al. 2016 stated that *Fusarium* sp. had the potential to control *Phytophthora capsici*, causing foot rot disease of black pepper. de Lamo and Takken (2020) revealed that nonpathogenic *Fusarium oxysporum* induced resistance against numerous fungal pathogens. Meanwhile, Sriwati et al. (2015) concluded that endophytic *Trichoderma virens* reduced the expansion of *P. palmivora* lesions on detached cocoa pods up to 77%. Similar finding was also informed by Abdallah et al. (2015), in which they examined nine isolates of *Aspergillus* spp., as biological control agents against *P. erythroseptica*, the causal organism of pink rot disease of potato.

Table 1. The percentage of inhibition and the mechanism of antagonism of endophytic fungi against *Phytophthora palmivora* in dual culture method.

Endophytic fungi	Mean % inhibition	Mechanisms of antagonism		
		Antibiosis	Competition	Myco-parasitism
*Aspergillus*1	58.98 cde		+	
*Aspergillus*2	57.58 cd		+	
*Aspergillus*3	79.33 g	+		
*Aspergillus*4	80.00 f		+	
*Aspergillus*5	88.67 i		+	
*Aspergillus*6	80.67 gh		+	
*Cylindrocladium*1	71.01 f		+	
*Cylindrocladium*2	47.33 b	+		
*Cylindrocladium*3	50.33 b	+		
*Fusarium*1	61.67 de		+	
*Fusarium*2	58.52 cd		+	
*Fusarium*3	56.54 c			+
*Fusarium*4	74.00 f	+		
*Fusarium*5	85.33 hi	+		
*Fusarium*6	88.83 i		+	
*Fusarium*7	80.60 gh		+	
*Gliocladium*1	73.77 f		+	
*Gliocladium*2	63.56 e			+
*Gliocladium*3	84.00 ghi		+	
*Mortierella*1	39.33 a		+	
*Mortierella*2	39.00 a	+		
*Mortierella*3	62.00 de	+		
Ramichloridium sp.	87.67 i		+	
Rhizoctonia sp.	60.50 cde	+		
Unknown	58.52 cd		+	

Note: superscript letters denote significant difference (LSD 0.05) among the treatment means.

Table 2. Spot diameter of cocoa pod after treatment with endophytic fungi following inoculated with *P. palmivora*.

Endophytic fungi	Spot diameter of cocoa pod (cm)
*Aspergillus*3	3.70 e
A*spergillus*4	2.03 ab
*Aspergillus*5	2.57 bc
*Aspergillus*6	1.80 a
*Fusarium*4	2.90 cd
*Fusarium*5	2.70 cd
*Fusarium*6	2.00 ab
*Fusarium*7	3.80 e
*Gliocladium*3	3.30 de
Ramichloridium sp.	2.73 cd
Control	6.47 f

Note: superscript letters denote significant difference (LSD 0.05) among the treatment means.

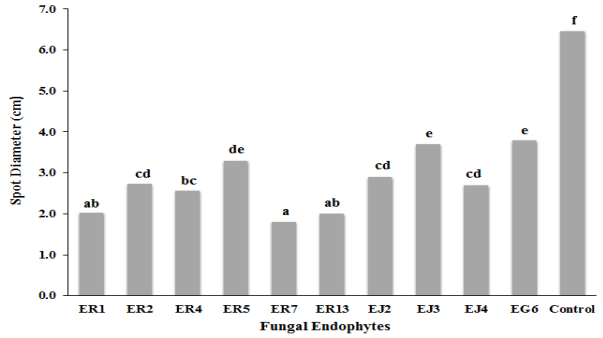

Figure 2. The effect of fungal endophytes against *P. palmivora* on cocoa pods (ER1=*Aspergillus*4, ER2= *Ramichloridium* sp, ER4=*Aspergillus*5, ER5= *Gliocladium*3, ER7= *Aspergillus*6, ER13= *Fusarium*6, EJ2= *Fusarium*4, EJ3=*Fusarium*5, EJ4= *Aspergillus*3, EG6= *Fusarium*7). Different superscript letters above the bar indicate statistically significant differences by LSD post hoc test (0.05).

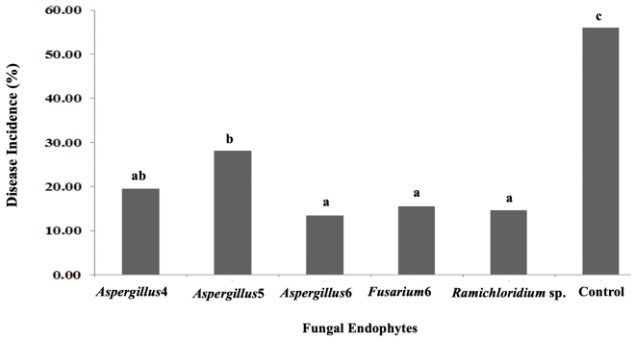

Figure 3. Mean disease incidence (%) of cocoa leaves inoculated with *P. palmivora* following the treatment of endophytic fungi. Different in letters above the bar indicate statistically significant differences by LSD post hoc test (0.05).

The ability of *Aspergillus* spp. to reduce the growth of *P. palmivora* lesions on detached cocoa pods is possibly due to their ability to produce a lot of derivative metabolites with various biological importance, such as antipathogens activities (El-hawary et al. 2020). Additionally, Myrchiang et al. 2014 reported that *Aspergillus fumigatus* secreted aflatoxin, carcinogenic and mutagenic secondary metabolites that inhibited the growth of *Phytophthora infestans in vitro*.

Screen-house experiment

All isolates tested on cocoa seedlings notably reduced the disease incidence of *P. palmivora* on leaves compared to untreated (uninoculated) seedlings (Figure 3). Figure 3 revealed that *Aspergillus*6 isolate showed maximum inhibition against *P. palmivora* but did not differ significantly with *Ramichloridium* sp., *Fusarium*6, and *Aspergillus*4 isolates. This result was slightly different from the pod trial. In the cocoa pod trial, *Ramichloridium* sp. isolate alone demonstrated lower inhibition percentage (Figure 2) than the screen house trial. This difference may be because *Ramichloridium* sp. had different antagonistic modes. So, it is helpful to properly recognize the plant-endophytes ecosystem model and classify the methods of collaboration of endophytes, plants, and phytopathogens to obtain practical biological control approaches (Mejía et al. 2008).

The first symptom on leaves was observed seven days after the inoculation of *P. palmivora*. Leaves had small necrotic spots with around 1 mm, and as the infections continued, the necrotic lesions and leaf blight became more apparent. No symptoms observed on the stem. The disease incidence on the untreated control seedling was 56%, and it significantly lower on the treated seedlings that ranged from 13.48% – 28.07%. In this seedling trial, pre-inoculation of endophytes was established to better colonization of the endophytes prior to the arrival of pathogenic *P. palmivora*. The activities of endophytes in limiting *P. palmivora* were confirmed by Mejía et al. 2008; Hanada et al. 2010; Sriwati et al. 2015; Harni et al. 2014. This experiment indicates that fungal endophytes obtained from infected cocoa pods restricted the growth of *P. palmivora*.

Field trial

Changes in disease severity of cocoa pods that occurred after application of endophytic fungi are presented in Table 4 and Figure 4. Table 4 shows that endophytic fungi application on cocoa pods did not reduce pod rot disease severity. Disease progression continues; even when cocoa pods were healthy at first, the disease can develop. There were many diseased pods in the garden so that the infection from the diseased fruit quickly spread to the healthy fruit. Even low severity fruit (10% and 25%) could rapidly increase in severity to 100% within four weeks. The disease severity is supposed to rely on three factors, the host plant, the pathogen virulence, and environmental conditions. Field conditions at the time of endophytic application favored the disease development (± 80% humidity and rainy day, temperature 24-29^{0}C in the second

week of May 2019). This result was confirmed by the earlier studies concerning the abitoc factors of *P. palmivora*'s growth and development. Zoospores of *P. palmivora* require approximately 20-30 minutes of free water on external plant membranes to propagate and enter the plant tissue (Guest 2007) as the optimum temperature for highest mycelial growth of *P. palmivora* ranges from 25-28˚C (Kudjordjie 2015). Moreover, Guest (2007) stated that *Phytophthora* spp. has a rapid life cycle, complicated disease cycle, zoospore motility, and a remarkable multiplicative ability to cause such a significant disease that is very challenging to be controlled by biological control agents. Nevertheles Guest (2007) also affirmed that antagonistic endophytes propose more guarantee as they are naturally transferred from adult plants to saplings in the natural cocoa plantations.

It is observable that endophytes can affect the disease severity. Specifically, they have been performed to reduce or intensify plant disease severity in functional assays that involve susceptible plants, infectious pathogens, and a favorable abiotic ecosystem (Busby et al. 2016). The relationship of plant-endophyte varies from mutualism to pathogenicity. Endophytes can possess neutral or harmful outcomes to the host plant under natural growth circumstances, though they can be valuable under more complicated situations or through several phases of the plant life cycle. Abiotic and biotic aspects influenced the effective colonization by endophytes, such as genotypes of plants and microorganisms, ecological situations, and the active web of collaborations within the plant biome (Hardoim et al. 2015; Chitnis et al. 2020). Our field result implied that we are at the very beginning of identifying the association of endophytic fungi- *P. palmivora* of cocoa and more diverse investigation settings would be required for greater understanding into endophyte performing.

In this trial, we did not perform a phytosanitary cleaning as conducted by Mejía et al. 2008, where all infected fruits were eradicated from the plants more than seven months before the fungal endophytes application. Destroying the primary inoculum sources inhibited the transmission of disease among the trees, reducing the production of secondary inoculum and spores' movement from the soil to the canopy (Guest 2007). It is implied that the cleaned farm with no pathogens and debris helps the endophytic fungi to perform their best antagonist activity against plant pathogen. This experiment was conducted in a small-scale plantation with a lack of sanitation, no pruning and weed control, inappropriate removal of pod mummies and diseased pods. This condition had provided a high inoculum level of *P. palmivora*, and the endophytic fungi did not have time to establish and protect the plant from the pathogen. This result was in agreement with the study from Hanada et al. (2009), which revealed that the utility of biocontrol organisms is connected to their ability to multiply and persist on the hosts for an extended time after treatment. On the other hand, Hanada et al. 2010 indicated that the grownup pods are possible no longer under the plant's systemic resistance fortification. This condition possibly assists an asymptomatic endophytic fungus to develop an aggressive rot-causing microorganism. This result suggested that the application of fungal endophytes to cocoa trees should be done more than once, from vegetative to generative phases. Besides, Guest 2007 confirmed that fungal endophytes ultimately vanish from the farms, so re-applied them into cocoa continue and secure tree against *Phytophthora*. Also, our screen house results suggested that fungal endophytes application in plants should be conducted before the arrival of pathogens. The capability of antagonists to multiply within a short period of favorable environmental conditions before confronting plant pathogens is an important factor that increases their efficacy in the field. Also, Mmbaga et al. (2018) claimed that plant inoculation with endophytes prior to the pathogens was more effective than introducing the pathogen before the endophytes.

Investigation on endophytic fungal population has been carried out in few plants (Gouda et al. 2016; Fadiji and Babalola 2020b). Hence, any studies or exploration or data regarding endophytic microorganisms-host relationship is worthy in this research. Additional taxonomic features on the five fungal endophytes relations with the plant are undoubtedly needed. Besides, the improvement of successful biological control approaches relies on diagnosing the relationship of plant endophyte pathogen, which is correlated to validating endophyte habits, settlement model, and spread mode (De Silva et al. 2019). Thus endophytes offer valuable natural resources for potential application as biocontrol agents.

Table 4. Disease severity of black pods before and four weeks after application of endophytic fungi.

Endophytic fungi	Disease severity before the application of endophytic fungi* (%)	Disease severity after the application of endophytic fungi* (%)
Control	0	20
Control	0	48
Control	0	36
Control	0	25
Control	0	42
*Aspergillus*4	>10≤25	50
*Aspergillus*5	>10≤25	50
*Aspergillus*6	>10≤25	80
*Fusarium*6	>10≤25	38
Ramichloridium sp.	>10≤25	50
*Aspergillus*4	>25<50	42
*Aspergillus*5	>25<50	80
*Aspergillus*6	>25<50	70
*Fusarium*6	>25<50	80
Ramichloridium sp.	>25<50	60
*Aspergillus*4	≥50	80
*Aspergillus*5	≥50	92
*Aspergillus*6	≥50	90
*Fusarium*6	≥50	100
Ramichloridium sp.	≥50	100

Figure 4. Disease severity of cocoa pods before (first row) and four weeks (second row) after application of endophytic fungi

This study suggested that the *Aspergillus*4, *Aspergillus*5, *Aspergillus*6, *Fusarium*6, and *Ramichloridium* sp. can be used to control cocoa black pod disease caused by *P. palmivora*. Different fungal endophytes exhibited potential as biocontrol agents against *P. palmivora* in dual culture, pod, and seedlings assays in the present experiment. However, these assays' result was not found effective in disease suppression on plants in the field. Further research on the application of endophytic fungi in the field needs consideration of phytosanitary aspects and the quantity of endophyte treatment.

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