Detection of phytopathogen and antagonistic fungi on the black diseased-cacao pod

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Abstract. Isolation of phytopathogen and antagonistic fungi is important in the efforts for managing phytopathogen infestation in cacao plantations. It is related to developing studies to evaluate the association between the two types of organism in the context of establishing integrated pest management to manage widespread pest and disease in cacao crop. This research objective is to study and isolate phytopathogens and antagonistic fungi on cacao. Fungal isolation was conducted from black diseased-cacao pod and soil samples through surface sterilization and serial dilution, respectively. After several culture purification, four fungal isolates were identified through macroscopic colonies and microscope observation. Those fungal isolates are Curvularia, Fusarium, Aspergillus and Trichoderma. Further research is necessary to test their antagonistic and or synergistic associations in laboratory and field scales.

1. Introduction

Indonesia is the third largest producer of cacao beans, after Ghana and Ivory Coast, with the production was up to 320,000 tonnes in 2015/2016, almost 10% of world’s total cacao beans production, yet, the production was estimated to decrease until 25% [1]. Central Sulawesi was the main cacao beans producer in 2016 accounted for 18.97% of total production in Indonesia and was predicted to keep the position in 2017, Meanwhile, the total of cacao product’s export in 2017 was preliminarily valued at US$ 1.12 billion [2].

Although cacao production is the top five of the largest foreign exchange earnings from the agriculture sector, it has been projected to decrease mainly due to the reduction of cacao cultivation area and diseases. Plant disease is the main contributor to cacao production loss rather than the infestation of arthropod and invertebrate pests. Major diseases in cacao tree (Theobroma cacao) that is responsible for great loss in annual production are mostly fungal infected disease, namely black pod, witches broom, frosty pod, and vascular streak dieback [3]. These fungal diseases have reduced cacao production by 40% worldwide [4, 5].

The black pod disease of cacao is caused by several species of Phytophthora that is not only affected cacao production but also resulted in tree debilitation and root infection on more severe level [3]. The pathogen attacks not only cacao pod but also other parts, although the incidence is less frequent, such as beans, flower cushion, leaf stem and root [6-8]. However, phytopathogen species, P. megakarya, was reported to only attack cacao pods [9]. Cacao fruit or pods can be infected by the pathogen from early to late developmental stages [10]. The infection starts from a small lesion spot then it spreads rapidly to all over the pod surface and invades internal tissue, thus in advanced stage may result in
disinfection of the cacao beans [4]. Fungal pathogen *P. palmivora* was thought to be one of cacao plantation major diseases in South East Asia [11]. Furthermore, a survey was conducted on a 400 ha cacao plantation of an assigned cacao center production located in Agam District, West Sumatera and found that 86% of rotten fruit sample was caused by *P. palmivora* [12]. A similar result was also reported previously in Bukittinggi lowland and highland cacao plantation, West Sumatera [13].

The control of black diseased cacao pod using copper based-fungicide is mostly used and relatively effective but faces environmental and health problems for illiterate or smallholders farmers who produce most cacao worldwide [7]. Moreover, the fungicide application is rarely cost effective due to fluctuating beans price [14]. Cultural practices to reduce the incidence of black pod disease through regular pruning, tree spacing and removal of the infected pod as inoculums source are also useful to avoid pathogen dispersal [15]. However, the method is labor intensive, and the efficacy relies on labor cost, frequency, and most important, the life cycle of the causal agent [3, 6, 16].

Using beneficial indigenous endophytic and epiphytic agent as a biocontrol agent for plant disease offers ecological benefits as those isolates are already being acclimated to the host and abundantly present at the site [17]. Beside *Trichoderma*, many other endophytic/epiphytic may also have the potential to antagonize *P. palmivora* [18,19]. Therefore, exploration and screening for other culturable local isolate are needed to be carried out. The purposes of this research are to isolate phytopathogens found on the diseased-cacao pod and explore the potential of the isolated antagonistic fungi.

2. Materials and method

2.1. Cacao pods

Cacao pods with black rot symptom and soil samples were collected from a cacao plantation in Ciampea, Bogor and Pathuk, Gunungkidul, Yogyakarta in August and November 2018, respectively. The samples were brought to the Laboratory of Microbiology, Research Center for Biomaterials-LIPI. Cacao pods were humidified for two days to trigger sporulation of *Phytophthora* on the outer fruit surface.

2.2. Soil samples

Three soil samples were only collected from cacao plantation in Ciampea, Bogor. These soil samples were then bagged and stored in room temperature (25-28) °C for five days.

2.3. Isolation of phytopathogens

Isolation phytopathogens were conducted in two approaches namely direct isolation from diseased cacao pod and indirect isolation from soil samples that were collected from under cacao trees which bear diseased cacao pods.

2.4. Direct isolation

Cacao pods were cut into approximately (2 x 2) cm² with a sterile scalpel; the tissue was then dipped 2.5% NaOCl for 1-2 minutes and finally rinsed with sterile distilled water. The tissue was subsequently cut into smaller pieces (3-5 x 3-5) mm² and then inoculated on *Potato Dextrose Agar* (PDA) in the Petri plate size (9 x 9) cm². The inoculated tissue was incubated in room temperature 25-28 °C for 5-8 days. Any fungal growth colonies were observed and a single culture method was used to purify more than one fungal colonies.

2.5. Indirect isolation

Dilution to extinction was used for isolating phytopathogens from soil samples. Three soil samples collected from three different spots in cacao plantation were thoroughly mixed. Twenty gram of mixed soil samples were put into Erlenmeyer flask filled with 190 ml distilled water to prepare a stock solution (100). Subsequently, aliquot 10 ml was pipetted out of stock solution and pipetted into new Erlenmeyer flask filled with 190 ml sterile distilled water continuously until it reached serial dilution 10-1, 10-2 to 10-9. This method is a modified version described by [20].
One milliliter solution of each Erlenmeyer flask was squirted, spread and plated on PDA agar in Petri plates. These treated Petri plates were incubated in room temperature 25-28 °C for 48 hours. Each concentration was plated with three replications.

2.6. Identification of isolated fungi
Isolated fungi were single cultured and maintained in PDA media for 3-5 days in room temperature 25-28 °C for identification. Fungal identification into genus level was done based on the description of macroscopic characters, spore-bearing structures (conidiophores) and spores.

Figure 1. Cacao pods without (left) and with (right) humidification.

Figure 2. Erlenmeyer flask filled with serial dilution with serial concentration $10^0$ to $10^{-9}$.

Figure 3. Incubation of Petri plates inoculated with a serial dilution of soil samples.

3. Results and discussion
In this study, two fungal genera were isolated with direct isolation method. Those fungi were a phytopathogen and one candidate for antagonistic fungus. The phytopathogen genus was *Curvularia* whereas the potential antagonistic genus was *Aspergillus*. 
Figure 4. From left to right: macroscopic appearance, spores and mycelium of *Curvularia*.

*Curvularia* is commonly found in tropical areas, but some species are discovered in temperate regions. According to [21], this fungal genus is facultative phytopathogen and especially fungal species. *Curvularia lunata* is known for causing leaf spot disease on cacao plants. Additionally, *Curvularia* is recognized as an endophytic fungal genus in the tissue of cacao plants and shows abilities in managing the infestation of *Phytophthora* beside *Trichoderma, Fusarium, Tolypocladium* and *Pestalotiopsis* [19]. Further molecular identification and evaluation tests on antagonistic association are necessary to determine the identity and role of *Curvularia* isolate found in this study.

Presumably, this is the first study that *Curvularia* was found in cacao pod in Indonesia beside the previous research that *Curvularia geniculata* was documented isolated from cacao seeds [22].

Figure 5. From left to right: macroscopic appearance and conidiophores of *Aspergillus*.

Figure 6. From left to right: a. *Fusarium* sp, b. *Trichoderma* sp, c. *Aspergillus* sp, d. unidentified fungus.

Figure 7. Inhibition reaction of *Aspergillus* against *Curvularia*. 
Some fungi were isolated from cacao pod collected from a plantation in Gunungkidul, Yogyakarta. Based on the macroscopic characters such as mycelium colony, spore-bearing structures and spores through microscope observation, these fungi are identified as *Fusarium* sp, *Trichoderma* sp and *Aspergillus* sp (figure 6).

Fungal genus *Aspergillus* was isolated un-intentionally as *Curvularia* was single cultured from cacao tissue. *Aspergillus* was found as a contaminant of *Curvularia* and then simply tested without replication. When the two fungi were grown together, it appeared that *Aspergillus* formed clear zones toward *Curvularia* (figure 7). The clear zones indicate potential inhibition activity between two fungal species. However, studies with replication are necessary to confirm the antagonistic pattern between the two genera.

Fungal isolation from soil samples with the dilution method did not have a significant result since no fungal strain was isolated. Causes as the justifications for this result can be many, for example the serial concentration and isolation media. Serial concentration in this method was 10-10^9 ml and used PDA as isolation media. This isolation method referred to [20]. Aside from increasing serial dilution concentration, the use of Rose Bengal Agar is better than PDA media if the target isolation is fungi [23].

Some methods have been studied to manage an infestation of phytopathogens of cacao crops especially towards genus *Phytophthora* [6]. However, other phytopathogens are worth to be studied as well as their association toward each other.

### 4. Conclusion

Some fungal genera indicated as phytopathogens were isolated from cacao pod. Those fungi are *Curvularia* and *Fusarium*. Other fungal genera were *Trichoderma* and *Aspergillus* that were potentially antagonistic fungal species. Further trials are necessary to confirm the antagonistic relationship between those isolated fungi.

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